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Research Article
Optimizing Enzyme-Linked Immunosorbent Assay Parameters for Chlamydial Abortion Diagnosis in Sheep

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Abstract

Background and Objective: Chlamydial abortion is one of the leading causes of reproductive loss in sheep, yet diagnostic accuracy remains limited due to suboptimal assay conditions. To address this gap, the present study aimed to optimize the conditions of an Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of chlamydial abortion in sheep, using TT and MM strains of Chlamydia previously isolated from affected animals. **Materials and Methods:** Yolk sac suspensions from 6-7-day-old chicken embryos infected with the causative agent of sheep enzootic abortion were used as starting material for antigen preparation. The MM strain was employed to develop an optimal method for the purification and concentration of antigens. Hyperimmunization schemes were designed using purified proteins in combination with biostimulants to obtain positive serum. Conjugates were prepared from isolated immunoglobulins, achieving an activity of 1:800. Immunoglobulins were purified by triple precipitation with saturated ammonium sulfate solution, followed by gel chromatography on Sephadex G-200 and identification by immunoelectrophoresis. Statistical analyses were performed using Student's t-test ($p \leq 0.05$) for continuous data and a one-sided Fisher's exact test ($\alpha < 0.05$) for comparing group efficacy. **Results:** The study established optimal contact parameters for antigen antibody interactions in the ELISA system. Results showed that effective binding occurred within 3 hrs at 37±1°C or 18 hrs at 4±2°C, while specific conjugate-antigen interaction was optimal for 1 hr at 37±1°C. These optimized parameters significantly enhanced assay sensitivity and reliability, providing reproducible detection of antibodies against the chlamydial abortion pathogen. **Conclusion:** This study developed an optimized ELISA protocol for the diagnosis of sheep enzootic abortion by refining antigen preparation, immunoglobulin purification and incubation conditions. The findings contribute to improving diagnostic accuracy and may serve as a foundation for developing more rapid and cost-effective diagnostic kits for field application. Future research should validate these optimized conditions across broader sheep populations and different chlamydial strains.

Key words: Causative agent, conjugates, enzootic abortion, immunoglobulin, small ruminants

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Chlamydia abortion in sheep, also known as enzootic abortion, caused by the bacterium *Chlamydia abortus*, is an infectious, chronically occurring disease of sheep. It is caused by an intracellular organism of the Chlamydiaceae family of the species *C. psittaci*[1-3]. The disease most often proceeds chronically and leads to abortions, that is, premature birth of dead or non-viable lambs. Sometimes (with the penetration of chlamydia into the blood) pneumonia, enteritis and polyarthritis occur[1,4,5].

Ovine enzootic abortion of sheep (OEA) is spreading worldwide, causing significant economic losses[6-9]. In particular, this problem is faced in Europe, Mexico, China, Iran, Iraq, Italy, Bangladesh, Spain, Poland and other countries[10-21].

Enzootic (chlamydial) abortion usually occurs during the last 2-3 weeks of pregnancy and is characterized by stillbirth and extensive inflammation of the placenta.

In addition, infection can result in the birth of fully developed stillborn lambs or weak live lambs that do not live longer than 48 hrs[22].

Also, with multiple births in infected females, it is often possible to observe the appearance of one dead and one or more weak or normal, healthy lambs. As a rule, the infection gets into a "clean" herd with infected repair animals and in the first year leads to a small number of abortions, followed by a "flurry" of abortions in the second year, when the disease affects up to 30% of females. The microbe is present in the secretions of the infected placenta and the entrance route for it into the body is the oral cavity. Sheep can remain carriers after an abortion. Sheep infected before five or six weeks of pregnancy terminate pregnancy at a late stage, but sheep infected after five or six weeks of pregnancy terminate subsequent pregnancies. Pathogenesis includes infection in susceptible animals and persistence of *Chlamydia abortus* in an unknown place of the body. Approximately after the 90th day of pregnancy, *Chlamydia abortus* multiplies in the placenta and then in the trophoblasts of the intercellular placenta. This process leads to a cascade of cytokines and chemokines with inflammation and thrombosis of placental vessels and subsequent abortion. Sheep placentitis is similar to the description of bovine placentitis. Microbes cause the expansion of trophoblasts, which can be seen with the help of special dyes, such as modified colors of Zil-Nielsen or Jimenez. Fetal lymphocytes and macrophages may be present in the liver, lungs and muscles[23-28].

Infected animals do not show clinical signs of the disease before the occurrence of abortion, although changes in behavior and vaginal discharge may occur in the last 48 hrs with the phase of rapid fetal growth, occurring around day 90 of pregnancy. By penetrating the placenta, bacteria cause a diffuse inflammatory reaction, thrombotic vasculitis and tissue necrosis. In cases of complications, signs of cerebral hypoxia are possible[29].

In sheep, a key diagnostic indicator is abortion at a late stage of pregnancy with the separation of necrotic membranes, while it is necessary to carefully differentiate the diffuse picture of necrosis with changes caused by *Toxoplasma gondii* (cotiledones only). Differentiation with other infections that may cause abortion, for example, brucellosis, coxiellosis or other bacterial pathogens (Campylobacter, Listeria, Salmonella) can be carried out through microscopic examination or cultivation, to improve the collected diagnosis of the causative agent of chain infection *Chlamydia* results spp., leading to interruption of the infection, new techniques were used, such as substrate amplification of specific conjugates of infected nucleic acids (PCR and sequencing)[30].

An alternative approach for detecting Chlamydia is amplification of chlamydial DNA using Polymerase Chain Reaction (PCR) and real-time PCR. However, it should be taken into account that cross-contamination between samples or exogenous contamination of samples in the field may occur. Furthermore, if PCR inhibitors are present in samples, there is a risk of false-negative results. Despite these caveats, real-time PCR is becoming increasingly widespread due to its ease of standardization and high speed. Recently, DNA microarray hybridization assay technology has been developed using the ArrayTube platform, which is very promising with regard to the direct detection and identification of microorganisms contained in clinical samples. A technique for using PCR in combination with the analysis of restriction fragment length polymorphism has been developed with the prospect of distinguishing naturally infected animals from vaccinated individuals (DIVA).

In this regard, the development of methods for optimizing ELISA (Enzyme-linked immunoassay) diagnostics of chlamydial abortion is relevant.

However, the fact that this disease was considered exotic in Mexico until May 2016 has become a factor in the spread of the disease in our country due to the lack of diagnostic methods. The purpose of the work is to work out the parameters of the ELISA formulation a method for diagnosing sheep enzootic abortion for the detection of antigen and diagnosis of chlamydial etiology disease.

MATERIALS AND METHODS

Ethical approval: Ethical approval for the conduct of this study was received by the Bioethics Commission of the Research Institute of Biological Safety Problems under the Scientific Committee of the Ministry of Education and Science of the Republic of Kazakhstan (KN MES RK) (Protocol No. 2 of May 1, 2021).

Study area: The study was conducted on the basis of the laboratory for the diagnosis of infectious Diseases of the Research Institute of Biological Safety Problems, Gvardeysky, Zhambyl Region, Kazakhstan, in the period from January to December, 2022.

Study animals: In the study, three sheep were used in the amount of 3 heads of the Dolan breed under the age of one year were used; the sheep did not have antibodies to infectious diseases, as well as to the strains MM and TT of the causative agent CAS.

The DCE of 6-7 days of age were used to cultivate the causative agent of CAS and obtain chlamydia-chlamydia suspensions to produce a specific antigen for hyperimmunization of animals. The DHE infection and incubation of the pathogen were carried out according to the procedure described above. In total, 16 series of specific antigens of the causative agent of CAS from the strain "MM" were prepared in the volume range from 10 to 123 cm^3.

Cultivation of the causative agent CAS in DCE: Chlamydia reproduce well on the membranes of the CE (chicken embryos) of chicken embryos; therefore, 6-7 day-old chicken embryos were used as a cultivation system, infecting them with strains of MM and TT of the causative agent CAS. The embryos infected with chlamydia were kept in a thermostat at 35°C for 12 days with daily ovoscopy. Infected embryos die 4-13 days after inoculation. High concentrations of elementary corpuscles are found in smears prepared from vascularized shells of YS. Pathogen-containing suspensions were prepared on a phosphate buffer from the YS, in the smears of which an accumulation of elementary bodies was detected for further work. During the experiment, three passages on 6-7 day DHE were performed to update the strains MM and TT of the causative agent CAS. The presence of chlamydia in the suspension was controlled by PCR using the CHLA- COM test system for the diagnosis of animal and bird chlamydia.

Obtaining the antigen of the causative agent CAS: The YS (yolk SAC) from chicken embryos infected with chlamydia was used as a raw material for cooking. Infected embryos were incubated at a temperature of 35°C for 12 days. After the death of the embryos, dissection was performed 4-12 days after infection and the LC was extracted. The extracted YS were washed twice with saline solution.

To prepare a 10% suspension containing the causative agent, CE DCE was crushed into pieces with an area of 0.5-1 cm^2 and crushed with a homogenizer. Then 9 cm^3 of saline solution was added to 1 g of the sample. To select the optimal method, various options for obtaining a specific chlamydia abortion antigen were used. The specific antigens of the sheep enzootic abortion were prepared using the 3 methods described below. According to the 1st method: A 10% causative agent-containing suspension was lyzed at -45°C and +24°C, then centrifuged at 4300 g, the precipitate was removed and brought to the initial volume.

According to the 2nd method: 10% pathogen-containing suspension was treated with two-fold lysis (- 45+24°C) and according to the 3rd method: 10% pathogen-containing suspension was treated with three-fold lysis (- 45+24°C)-45°C and +24°C), the obtained sedimentary materials were brought to the initial volume and used as a specific chlamydia antigen. A positive (antichlamydial) serum was taken by administration of a specific (chlamydial) antigen to experimental animals sheep. The optimal dose of the produced immunoglobulin was calculated by direct ELISA by titration using specific (chlamydial) and normal (control) antigens of the pathogen of sheep enzootic abortion. Then the prepared antigens were inactivated with 0.5% solution of 98% beta-propiolactone for 48 hrs at a temperature of 37°C. The completeness of inactivation of specific antigens was checked by infecting 6-7 day DCE (incubation at 37°C for 12 days), followed by polymerase chain reaction (PCR).

Purification and concentration of the causative agent-containing suspension: When working out methods for purification and concentration of the pathogen of sheep enzootic abortion, the following methods were used: according to the 1st method, a liquid-containing suspension of YS of DCE was boiled at 100°C for 30 min; after cooling, 1:10 diethyl ether was added. The resulting suspension was left in the refrigerator at 4°C for 1.5-2 hrs after careful mixing. The ether fraction was mixed with the secondary extraction diethyl ether of the remaining suspension. According to the 2nd method, the ether from the resulting supernatant was removed in a water bath at 37°C with gradual cooling.

Hyperimmunization of sheep: Positive (antichlamydial) serums were produced in 2 ways. Experimental sheep were used as donors, who were immunized with a specific (chlamydia) antigen into the femoral region intramuscularly at a dose of 1.0 cm^3 (antigen concentration is equal to 212 mcg/cm^3), reimmunization was carried out 10 and 18 days after the first immunization by injecting chlamydia antigen with the adjuvant Montanide ISA-71 3:76 also into the thigh area of sheep. After the last hyperimmunization, after 7-14 days, blood serums were taken from sheep and examined for the presence of antichlamydial antibodies in DPR. The isolation of immunoglobulins from sera using the Kohn method was carried out in 3 ways: Precipitation of gamma and betaglobulins and separately beta-globulin and gamma globulin. Using the CLA1135T IDEXX ID Chlamydia Total Ab kit (serum or plasma kit), the level of formation of antichlamydial antibodies was studied. Also, in parallel, the presence of antibodies in the sera of immunized animals was checked by using the CLA1135T IDEXX ID Chlamydia Total Ab kit.

Clarification of the optimal dose of immunoglobulin. It was determined that 0.01 M carbonate-bicarbonate buffer (CBB) with a pH of 9.5 is the optimal solution for sensitization of the wells of the tablet and 0.01 M fetal bovine serum (FBS) with a pH of 7.4 containing Twin-80 in concentration is also acceptable for dilution of the components of the ELISA test system in the diagnosis of sheep enzootic abortion1%. To block the free centers of the tablet wells and remove the nonspecific background signal after sensitization with CAS immunoglobulin, the wells were treated with a 1.0% solution of bovine serum albumin prepared with 0.01 M FBS.

Clarification of the optimal dose of immunoglobulin was carried out by stepwise titration with specific (chlamydial) and normal (control) antigens in a direct ELISA test. For this purpose, the immunoglobulin was diluted with 0.01 M KBV with a pH of 9.5 in concentrations of 10, 25, 50, 100, 200, 300, 400 and 500 mcg/mL. Preparation of immunoperoxidase conjugates consisted of the following stages:

- Activation of horseradish peroxidase (HRP) by sodium periodate
- The process of labeling (conjugation) of gamma globulins with activated peroxidase;
- Purification of the obtained immunoperoxidase conjugate by gel filtration through Sephadex G-200

Then 16 mg of HRP (with a purity index RZ = 2.7-3.0 and a specific activity of 850 or more units of 4-aminoantipyrine) was dissolved in 4 cm^3 of distilled water. After that, 0.8 cm^3 of a freshly prepared 0.1 M NaIO4 solution was added to it; The resulting solution was stirred for 20 min at room temperature, then 6 drops of ethylene glycol were added. The resulting mixture was kept at room temperature for another 5 min. The peroxidase treated in this way was subjected to dialysis against 0.01 M KBB, with a pH of 9.5, for 18-24 hrs at a temperature of 4±2°C. 28 mg of gamma globulin was added to this fraction; then it was dialyzed against 0.01 M CBB, with a pH of 9.5, at a temperature of (4±2)°C for 18-24 hrs. After bringing the pH of 1 M CBB to 9.5, the reaction mixture was incubated for 3 hrs at room temperature and stirred with a magnetic stirrer. Then 0.32 cm^3 of NaVN4 was added (4 mg of NaVN4 was dissolved in 1 cm^3 of distilled water) and incubated under the same conditions for another 2 hrs. The resulting immunoperoxidase conjugate was dialyzed against 0.01 M FBS with a pH of 7.4 for 18-24 hrs at a temperature of 4±2°C.

The purified conjugate was purified from free unbound peroxidase on Se-notes G-200 (on a 1.2×100 cm column) balanced with 0.01 M FBS with a pH of 7.4; then it was collected in fractions of 3-5 cm^3 at an elution rate of 15-20 cm^3/hr (when cooling the column to 4-8°C and providing protection from direct sunlight rays). The collected fractions were monitored on a spectrophotometer at wavelengths of 280 and 403 nm.

For each conjugate fraction, the RZ index was calculated using the following formula[30]:

$$RZ = D403/ D280 \quad (1)$$

Fractions with RZ = 0.3-0.6 were collected into one conjugate and the rest were discarded.

The activity and specificity of immunoperoxidase conjugates obtained were tested using ELISA.

In addition, antibodies in blood sera were examined using the CLA1135T IDEXX ID Chlamydia Total Ab Kit (a set of sera or blood plasma).

In addition, the presence of antibodies to the causative agent CAS in animal blood sera after immunization was confirmed using CLA1135T IDEXX ID Chlamydia Total Ab Kit. For this purpose, the following buffer solutions were prepared: 0.1 M CBB, a solution for ELISA, a filler solution for ELISA, 0.15 M NaCl solution (saline solution), 0.01 M sodium acetate solution with a pH of 4.3-4.4%, a solution of hydrogen peroxide (H2O2); and the substrate solution for ELISA: 2.2 mg of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.1 cm^3 of 1% H_2O_2 dissolved in 10 cm^3 of 0.01 M sodium acetate.

In experiments on the development of a direct variant of the ELISA method for the diagnosis of HAC, various temperature-time modes of sensitization of plates with diagnostic drugs, the interaction of reaction components and

the time required to detect antigens using various buffer systems were tested. Various salt solutions were tested to establish the reaction and detect the antigen of the causative agent, CAS.

The optimal conditions for the formulation of the ELISA method for the diagnosis of sheep enzootic abortion have been determined. The optimal time and temperature parameters of interaction with the solid phase of the ELISA test in several stages have been established. Studies have been conducted to establish the optimal (for solid-phase sensitization) concentration of immunoglobulin and the working composition of the conjugate, as well as the effect of various conditions for setting the ELISA test on its sensitivity in the laboratory diagnosis of sheep enzootic abortion.

In the course of determining the conditions of implementing EIA in the diagnosis of CAS, flat-bottomed plates manufactured by foreign companies from Finland, Italy and the USA ("Costar") were used.

In experiments on the sensitization of plate wells with specific immunoglobulins of CAS, the following solutions were tested:

- 0.01 M CBB with a pH of 9.5
- 0.01 M FBS with a pH of 7.4

The following buffer solutions were tested to prepare working dilutions of EIA diagnostic preparations and to wash the plate wells:

- Saline solution containing Tween-80 in various concentrations (0.5, 1, 1.5%)
- 0.01 M FBS with a pH of 7.4, containing Tween-80 in various concentrations (0.5, 1, 1.5%)

Additionally, for clogging the free centers of the plate wells, after their sensitization with immunoglobulins, 0.5, 1.0 and 1.5% concentrations of bovine serum albumin in 0.01 M FBS solution were tested.

The scheme of implementing EIA for CAS included the following stages. A polystyrene flat-bottomed plate was sensitized with specific immunoglobulin in a working dilution on a solution of 0.01 M CBB with a pH of 9.5 for 18 hrs at 4°C. Then, a filler solution of 0.1 cm^3 in each well was introduced into the plate and incubated for 1 hr at 37±1°C. To assess the performance of the EIA, control antigens were used in consecutive double dilutions, starting from 1:10 on the EIA solution. All antigens were introduced at volumes of 0.1 cm^3. The plate with antigens was incubated for 18 hrs at 4°C. A specific conjugate at a volume of 0.1 cm^3 was introduced into each well in a working dilution (on a solution for EIA) and left in contact for 1.0 hrs at 37±1°C. Finally, 0.1 cm^3 of the substrate solution was introduced into each well and incubated for 30-60 min at room temperature.

Statistical analysis: The average values of the body temperature of the animals, serology data and clinical signs of animal disease were calculated with the removal of the standard error. The differences were calculated using Student's t-test, where $p \leq 0.05$ was considered significant. The difference in efficacy between groups was compared using a one-sided Fisher's exact test in two proportions at a significance level of alpha <0.05.

RESULTS

Chicken embryo susceptibility to chlamydia: The results of the conducted studies of the susceptibility of chicken embryos to chlamydia are presented in Fig. 1 and Table 1.

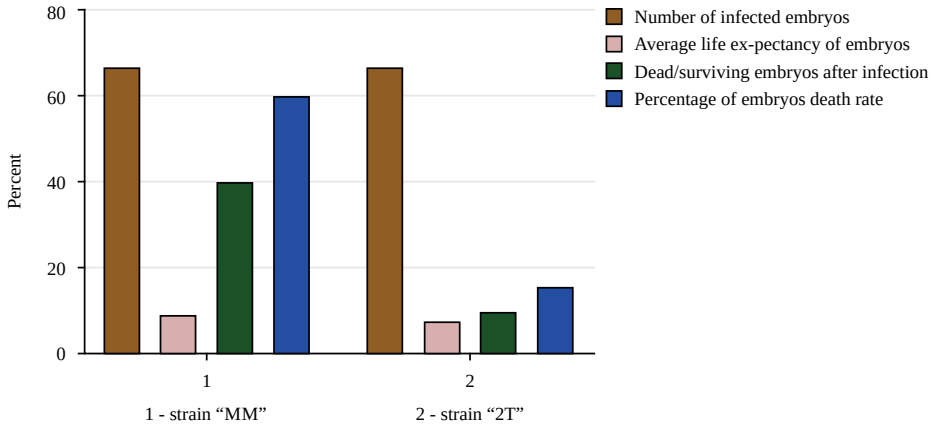


Fig. 1: Susceptibility of DCEs to sheep chlamydia abortion causative agent strains

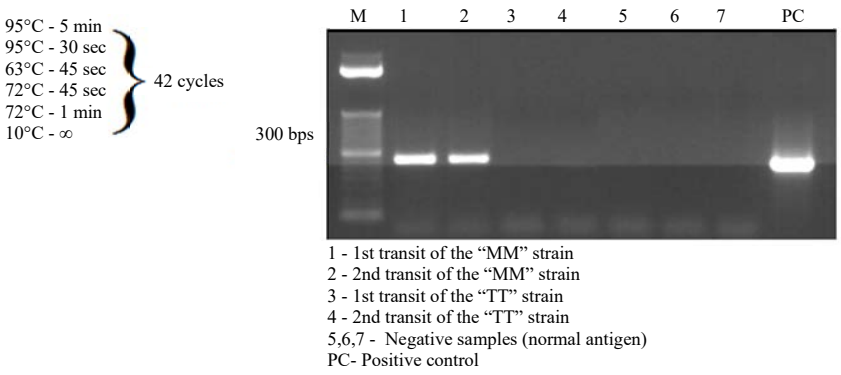


Fig. 2: Results of renewal of the CAS strains

Table 1: Biological activity of pathogens of CAS

Strains	Biological activity of l g/ ELD50/cm^3	Incubation periods (Days)
MM	5.12±0.045	12
TT	0	12

Table 2: Activity and specificity of the prepared antigens of the CAS pathogen

	Results in the DPR	
Studied antigens	PS	NS
Specific antigen No. 1	1:2	-
Specific antigen No. 2	1:2	-
Specific antigen No. 3	1:8	-
Normal antigen	-	-

-: Negative result, PS: Positive serum and NS: Normal serum

Figure 1 presents a side-by-side view of the MM and TT strains, showing (for each) the number of infected embryos, the average post-infection life expectancy, the counts of dead versus surviving embryos and the embryo death rate (%).

Table 1 reports the measured values: The MM strain showed a biological activity of 5.12±0.045 log ELD50/cm^3, while the TT strain showed 0; the incubation period was 12 days for both strains.

According to the results of Table 1, the MM chlamydia strain led to the deaths of 20 embryos 8-9 days after infection; 40 out of 50 embryos in the second transit died after 5-10 days and 60 out of 100 infected embryos in the 3rd transit died after 5-11 days. In addition, 7 out of 50 embryos infected with the TT strain of chlamydia died within 6-9 days and 10 out of 50 embryos died within 5-9 days in the 2nd transit; 100 embryos were infected in the 3rd transit and 13 of them died 4-12 days after infection. The embryos that had died 12 days after infection with the two strains of chlamydia were placed in a house gold refrigerator; after dissection, tissue smears were prepared from the extracted YSs. Stained smears of YSs were examined under a light microscope.

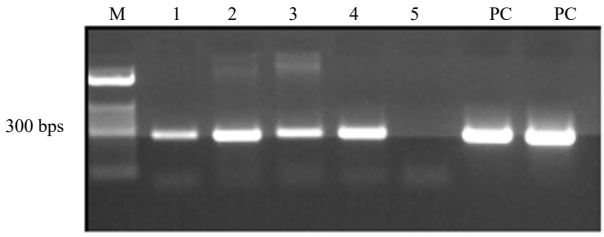
As a result of a microscopic study from smears of the YSs of DTEs infected with the "TT" strain, the causative agent of CAS was detected in the form of small microorganisms similar to cocci, while chlamydia was not extracted from the YSs of DTEs infected with the MM strain.

Thus, summarizing the data gathered from the experiments, it can be concluded that during three consecutive transits conducted with infection of DCEs, the MM strain of chlamydia showed infectious properties in the infected embryos with a fatal outcome and infectious properties were not detected for the embryos infected with the TT strain after three transits. Subsequently, experiments were carried out to determine the biological activities of chlamydia strains extracted from a suspension containing the causative agent prepared from the infected YSs of DTEs, the results of which presented in Table 2.

Table 1 shows that the biological activity of the MM strain of the causative agent of CAS for 12 days equaled 5.12±0.045 l g/ELD50/cm^3. We were unable to renew the TT strain experimentally.

The results of the detection of chlamydia using PCR are presented in Fig. 2 and 3.

Figure 2 and 3 present PCR results for a ~300-bp amplicon. In Fig. 2, lanes 1-2 (MM, 1st-2nd transit) and the positive control (PC) show clear ~300-bp bands, whereas lanes 3-4 (TT, 1st–2nd transit) and lanes 5-7 (negative samples) show no band.



1 - Source material of the "MM" strain
2 - 1st transit of the "MM" strain
3 - 2nd transit of the "MM" strain
4 - 3rd transit of the "MM" strain
5 - Negative sample (normal antigen)
PC- Positive samples

Fig. 3: Presence of sheep enzootic abortion n the causative agent-containing suspension

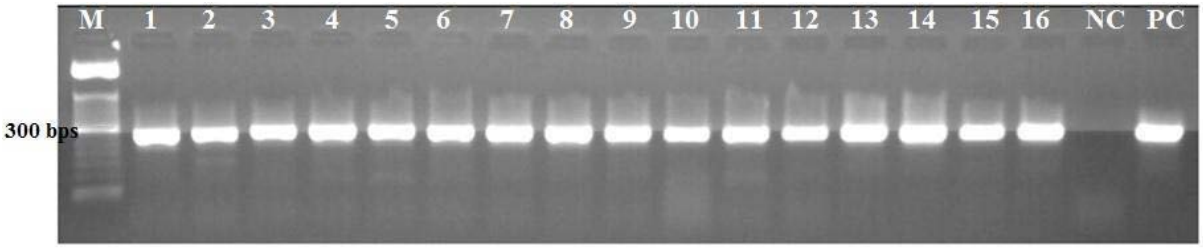


Fig. 4: Study of prepared antigens for the presence of DNA of the causative agent of CAS, Lane 16% prepared specific antigens of the causative agent of CAS, NC is the antigen and PC is the positive control sample

Table 3: Optimal methods for isolation and purification of Chlamydia, the causative agent of sheep enzootic abortion (n = 3)

Name of the virus-containing suspension	Activity in the DPR	CAS purifying method	Activity of purified protein in the CFT	Protein content (mcg/mL)
YS	1:8	First	1:16	532-970
YS	1:8	Second	1:8	250-432

In Fig. 3, the MM source material and 1st-3rd transits (lanes 1-4) and the positive controls (PC) display ~300-bp bands, while the negative sample (lane 5) does not. Lane M is the size marker; cycling parameters are indicated on the Fig. 3.

The data from the PCR study showed that, in suspensions of the MM strain of the first and second transits on the DCE, deoxyribonucleic acid (DNA) of the causative agent of CAS was present; meanwhile, in suspensions of the TT strain, there was no DNA of the causative agent of CAS. The presence of DNA of the causative agent of CAS in suspensions from YSs infected with the MM strain was comparatively assesses after three consecutive transits. As a result, the presence of DNA of the causative agent of CAS was discovered in all samples.

Preparation of the antigen of the causative agent of CAS: The activity of the prepared antigens was studied in DPR. The results of these studies are presented in Table 2.

Table 2 reports the DPR results for prepared antigens: Specific antigen No. 1 = 1:2 (PS),-(NS); Specific antigen No. 2 = 1:2 (PS), -(NS); Specific antigen No. 3 = 1:8 (PS), -(NS); Normal antigen = -(PS), -(NS).

Figure 4 shows PCR screening of the prepared specific antigens (lanes 1-16), with NC (negative control) and PC (positive control); the gel indicates a ~300-bp target band, lane identities as labeled on the Fig. 4.

The DNA of the causative agent of CAS was not detected in the studied samples of the prepared specific antigens, which indicates the completeness of the inactivation of specific antigens of the causative agent of CAS by the above-mentioned inactivation procedure. As a result of the conducted study, it was found that all the methods we tested for isolating and purifying the causative agent of CAS showed positive results. The activity of purified antigens in the CFT ranged from 1:8-1:16 (Table 3).

Table 3 summarizes the purification results from YS suspensions. For both entries, the activity in the DPR is 1:8. Using the First purifying method, the activity of purified protein in the CFT is 1:16 with a protein content of 532-970 mcg/mL. Using the Second method, the activity in the CFT is 1:8 with a protein content of 250-432 mcg/mL.

Table 4: Scheme of the hyperimmunization of sheep and the activity of the obtained antisera in serological reactions

		Dose of the material, concentration and frequency of protein administration			Activity in			
					DPR		CFT	
Animal	Injection materials	Dose (cm^3)	Concentration (mcg/cm^3)	Multiplicity	AgS	AgN	AgS	AgN
Scheme No. 1								
Sheep No. 1	Purified antigen No. 1	5	234	1	1:8	-	Unknown	Unknown
Scheme No. 2								
Sheep No. 2	Purified antigen No. 1	5	578	1	1:2	-	Unknown	Unknown

-: Negative result, AgS: Specific antigen and AgN: Normal antigen

Table 5: Specific activity of immunoglobulins isolated using various methods and the conjugates prepared on their basis in DPR and EIA

	Activity in the DPR			
Method of isolation of immunoglobulin	Initial serum	Immunoglobulin	Conjugate titter in EIA	Nonspecific background staining
Deposition with a saturated solution of $(NH_4)_2SO_4$	1:8	1:32	1:800	Weak
Alcohol deposition according to Kohn	1:8	1:32	1:800	None

Hyperimmunization of sheep: Producing a positive (antichlamydine) serum. Table 4 shows the immunization regimens of sheep, the activity in the produced antichlamydia-positive serum.

Table 4 summarizes two hyperimmunization schemes in sheep. Scheme 1: purified antigen No. 5, dose 1 cm^3, protein concentration 234 mcg/cm^3, single administration; DPR activity AgS = 1:8, AgN = -; CFT entries marked "unknown." Scheme 2: purified antigen No. 5, dose 1 cm^3, protein concentration 578 mcg/cm^3, single administration; DPR activity AgS = 1:2, AgN = -; CFT entries marked "unknown." Notes indicate "-" = negative, AgS = specific antigen, AgN = normal antigen.

The data in Table 4 indicate the greater importance of the first immunization regimen of experimental animals, in which an active antichlamydia serum was produced, the activity of which is in DPR-1:8 and CFK-1:48. In the diagnosis of the causative agent of sheep enzootic abortion, a direct dependence of the sensitivity, specificity and reproducibility of ELISA results on the quality of antibody conjugates with enzymes was established. In turn, the quality of conjugates depends on the activity, specificity and purity of immunoglobulins or antibodies used for conjugation, i.e. there is a self-dependence of all components of the ELISA test used in the diagnosis of sheep enzootic abortion.

Isolation of immunoglobulins: The isolation of immunoglobulins was carried out from a PS to the MM strain of the causative agent of CAS obtained according to the first scheme. The results of these experiments are presented in Table 5.

Table 5 compares two immunoglobulin isolation methods. With ammonium sulfate precipitation, the DPR activity is 1:8 for the initial serum and 1:32 for the isolated immunoglobulin; the ELISA conjugate titer is 1:800 and nonspecific background staining is noted as weak. With alcohol deposition by the Kohn method, the DPR activity is 1:8 (initial serum) and 1:32 (immunoglobulin); the ELISA conjugate titer is 1:800 and nonspecific background staining is none.

The results shown in Table 5 give reason to believe that the immunoglobulins produced by the Kohn method, using ammonium sulfate and alcohol treatment, turned out to be active and specific conjugates were developed. The activity of specific conjugates showed titers of 1:400-1:800; and four-fold working titers were 1:100-1:200. In a comparative study, nevertheless, the Kohn method of isolating immunoglobulin by precipitation with alcohol turned out to be of higher quality, since this method revealed a higher protein yield, because the prepared conjugate from immunoglobulins by isolation with ammonium sulfate gave a negative result in the form of nonspecific background staining in reaction with control (negative) antigens.

Determination of optimal assay conditions: To carry out diagnostic studies for the detection of the causative agent of CAS by the ELISA method, various salt solutions were worked out in order to determine the optimal temperature and time parameters of the interaction of components and sensitization of tablets included in the test system for ELISA diagnostics of CAS.

In our experiments, the optimal conditions for the contact of antigens with immunoglobulins in the diagnosis of CAS were 3 hrs at 37±1°C in the thermostat or 18 hrs at 4±2°C, as well as the contact of a specific conjugate with the antigen for 1 hr at 37±1°C. It was experimentally established that the optimal incubation mode of the peroxidase substrate (ABTS) with conjugates for weakly positive samples was 30 min. The results of the selection of optimal plates for use in EIA for the diagnosis of CAS are presented in Table 6 and Fig. 5.

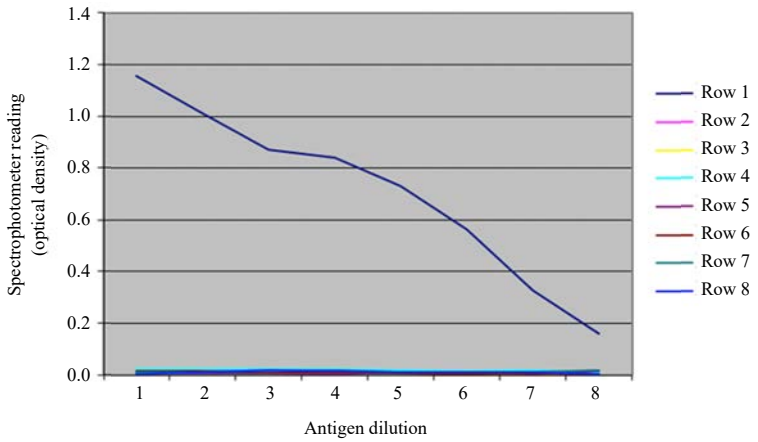


Fig. 5: Indicators of the optical density of specific and normal antigens of the causative agent of CAS in the setting of EIA

Row 1: Positive antigen of chlamydia abortion of sheep, Row 2: Negative antigen, Row 3: Positive sheep pox antigen, Row 4: Positive antigen of contagious ecthyma of sheep, Row 5: Positive bluetongue antigen, Row 6: Positive antigen of distemper of small ruminants, Row 7: Positive canine distemper antigen and Row 8: Positive brucellosis sheep antigen

Table 6: Indicators of the optical density of specific and normal antigens of CAS in the formulation of EIA using various flat-bottomed plates

		Dilution of antigens and optical density indicators							
Plate manufacturer	Antigens	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Finland	Specific	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01
	Normal	0.00	0.01	0.02	0.01	0.01	0.01	0.01	0.00
Italy	Specific	0.02	0.02	0.01	0.01	0.01	0.00	0.00	0.01
	Normal	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.01
USA "Costar"	Specific	2.94	2.93	2.96	2.89	2.90	2.92	2.90	2.91
	Normal	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.00

Figure 5 plots spectrophotometer optical density versus antigen dilution (1-8). The curve decreases from ~1.2 at dilution 1 to ~0.1 at dilution 8. Axis labels are "Spectrophotometer reading (optical density)" and "Antigen dilution." The legend/notes list antigens for Rows 1-8 as labeled on the Fig. 5: Row 1 positive chlamydia antigen of sheep; Row 2 negative antigen; Row 3 positive sheep pox antigen; Row 4 positive antigen of contagious ecthyma of sheep; Row 5 positive bluetongue antigen; Row 6 positive antigen of distemper of small ruminants; Row 7 positive canine distemper antigen; Row 8 positive brucellosis sheep antigen.

The data shown in Table 6 and Fig. 5 indicate that polystyrene plates manufactured in the USA were the most sensitive plated for performing EIA for the diagnosis of CAS to detect antigens, since active and specific results were obtained using these plates. The plates from Finland and Italy proved to be only slightly effective for use in EIA for the diagnosis of this disease. The results of studies on the selection of the optimal concentration of immunoglobulins (for the sensitization of plates) for EIA for the diagnosis of CAS are presented in Table 7.

Table 7 lists ELISA outcomes (+/-) for an immunoglobulin with a DPR titer of 1:32 tested at 10, 25, 50, 100, 200 and 300 mcg/mL. For the specific CAS antigen, all concentrations were positive (+) across dilutions 1:10-1:640. For the normal antigen, results were negative (-) at dilutions 1:10 and 1:100 for concentrations 10-200 mcg/mL; at 300 mcg/mL, dilution 1:10 remained-, while dilution 1:100 was+.

Thus, from the results shown in Table 7, it can be seen that all tested concentrations of CAS immunoglobulin with an activity in DPR of 1:32 produced a positive reaction in EIA with a specific antigen and a negative reaction with a normal antigen up to a concentration of 200 mcg/mL. The maximum concentration of immunoglobulin that made it possible to detect the specific antigen in its maximum dilution was 100 mcg/mL.

An 8-fold limit titer should be used as a reliable conjugate titer when setting up ELISA diagnostic studies to detect the causative agent of sheep enzootic abortion disease. In this case, the limit titer of the conjugate is determined by conducting a direct variant of ELISA. The limiting titer of the conjugate is considered to be its maximum dilution, at which a chlamydial antigen is detected and a negative result is taken with a normal (control) antigen.

Table 8 shows the average data of the studies conducted to determine the working dilution of a specific conjugate in the diagnosis of CAS.

Table 7: Selection of the optimal concentration of immunoglobulin

Immunoglobulin		Dilution of specific CAS antigen							Dilution of normal antigen	
Titer in the DPR	Tested concentrations, mcg/mL	10	20	40	80	160	320	640	10	100
	10	+	+	+	+	+	+	+	-	-
	25	+	+	+	+	+	+	+	-	-
	50	+	+	+	+	+	+	+	-	-
1:32	100	+	+	+	+	+	+	+	-	-
	200	+	+	+	+	+	+	+	+	-
	300	+	+	+	+	+	+	+	+	+

Dilutions of antigens are expressed in inverse values, +: Positive result in EIA and -: Negative result

Table 8: Determination of the working dilutions of a specific conjugate

	Dilution of specific antigen							Dilution of normal antigen	
Conjugate dilution	10	20	40	80	160	320	640	10	100
50	+	+	+	+	+	+	-	-	-
100	+	+	+	+	+	+	-	-	-
200	+	+	+	+	+	-	-	-	-
400	+	+	+	+	-	-	-	-	-
800	+	+	-	-	-	-	-	-	-
1600	-	-	-	-	-	-	-	-	-
3200	-	-	-	-	-	-	-	-	-
6400	-	-	-	-	-	-	-	-	-

Dilutions of conjugates and antigens are expressed in inverse quantities, +: Positive result in the EIA and -: Negative result

Table 8 lists ELISA outcomes (+/-) for a specific conjugate tested at dilutions 1:50-1:6400 against specific and normal antigens. With the specific antigen, conjugate dilutions 1:50, 1:100, 1:200 and 1:400 are positive (+) across all antigen dilutions 1:10-1:640. At 1:800, only antigen dilution 1:10 is positive, while 1:20-1:640 are negative (-). Conjugate dilutions 1:1600, 1:3200 and 1:6400 are negative at all antigen dilutions. With the normal antigen (dilutions 1:10 and 1:100), results are negative for all conjugate dilutions.

From the data shown in Table 8, it can be seen that the limit conjugate titers for the diagnosis of CAS using EIA were 1:800. Hence, the 8-fold working dilution of the conjugate was 1:100.

DISCUSSION

Sheep chlamydia, also known as enzootic sheep abortion, is caused by the bacterium *Chlamydia abortus*. Chlamydia abortion in the late pregnancy is the cause of the high mortality rate of fetuses and newborn lambs in many sheep-breeding regions of the world, especially in the case of high overcrowding of flocks during the lambing period[31-33].

In sheep, late gestation abortion with the separation of necrotic membranes is a key diagnostic indicator and the diffuse pattern of necrosis must be carefully differentiated from the changes associated with Toxoplasma gondii (cotyledon only). Differentiation from other infections that can cause abortion, such as brucellosis, coxiellosis, or infection with other bacterial pathogens such as Listeria and Salmonella, can be achieved through microscopic examination or culture[34].

Until recently, complement binding and long-term binding reactions (CFT and PCFT) were used in laboratories to diagnose SEA, however, antibodies against related pathogens of the genus chlamydia were not always detected in these reactions (for example, between *Chlamydia abortus* and *C. Pecorum*), there is no connection between sick and vaccinated animals and low titers of these reactions were also noted in a separate animal and a healthy herd, where abortions have not been registered before. Scientists have developed various serological methods and their variants, but they also had both good diagnostic efficacy and undesirable test results in the diagnosis of sheep enzootic abortion, some tests had commercial purposes, sometimes sheep infected with the sheep enzootic abortion pathogen showed delayed hypersensitivity to the antigen of the disease and this procedure is not suitable for normal practice. Various anti-chlamydia vaccine preparations have also been developed and are being used in practice, the timely use of which prevents abortions with chlamydia etiology, the spread of the pathogen in the environment and among herds of animals, but do not always give results of complete obsolescence of the disease. And therefore, periodic serological testing among the aborted herd of animals for the presence of the causative agent of sheep enzootic abortion provides a clear idea and the possibility of proper planning of anti-chlamydia measures in the fight against this abortifacient infection[35,36].

There are commercial versions of several tests for the determination of generic chlamydia antigens. In Ireland, scientists compared three commercial ELISAs in terms of their sensitivity and assessed the effect of different antigens used in the assays on test performance.

Comparative evaluation of some of these tests on nonsheep material has shown that ELISA-based tests are more sensitive that CFT kits. Under the conditions of the test, the most sensitive of the ELISA-based emergency diagnostic systems included in the study turned out to be a kit for the determination of chlamydial lipopolysaccharide (LPS).

It should be noted that the set under consideration does not allow for differentiation between *C. abortus* and other types pf chlamydia that can infect samples. In histopathological sections, antigen detection is performed using commercially available antibodies to the LPS or OMP (major outer membrane protein) of bacteria. The ELISA techniques developed independently by several groups of researchers have not been adapted for general diagnostic work.

In this research, we accounted for the above problems when developing methods for optimizing the ELISA diagnosis of chlamydial abortion in sheep. The difference between our studies and those mentioned above is that we obtained a specific antigen from a local strain of the chlamydia pathogen; as a component for inclusion in the ELISA kit, serum positive for the causative agent of chlamydia abortion in sheep was obtained. As a result of our studies on DCEs, it was found that the biological activity of the MM strain of the causative agent of CAS after 12 days of cultivation was 5.12±0.045 l g/ELD50/cm^3.

CONCLUSION

This work delivers a robust ELISA for detecting *Chlamydia abortus* antigen in small ruminants. Among candidate strains, MM consistently demonstrated biological activity whereas TT did not, establishing MM as the reliable source material. Antigen preparations achieved diagnostically useful activity (1:8-1:16 in DPR) and hyperimmune sera provided serological activity of 1:16-1:32. The Kohn alcohol method yielded a stable immunoglobulin fraction that enabled an HRP conjugate performing reliably at 1:800 in ELISA. Assay performance was maximized with 100 µg/mL capture immunoglobulin on high-quality U.S.-made polystyrene plates, with flexible incubation windows (3 hrs at 37°C or overnight at 4°C) and a 30 min ABTS readout, which together supported detection of weakly positive samples. These outcomes translate into a practical, scalable test system backed by approved regulatory/technical documentation and ready for routine veterinary diagnostics. By improving analytical sensitivity and workflow flexibility, the assay can facilitate earlier case identification and strengthen control programs for enzootic abortion of ewes in the republic and beyond.

SIGNIFICANCE STATEMENT

This study discovered the optimized ELISA conditions for detecting antibodies against chlamydial abortion in sheep, which can be beneficial for improving diagnostic accuracy and establishing reliable field-ready detection systems. By refining antigen preparation, immunoglobulin purification and incubation parameters, the study provides a practical framework for developing rapid and cost-effective diagnostic kits. This study will help researchers uncover the critical areas of antigen-ntibody interaction dynamics that many were not able to explore. Thus, a new theory on ELISA optimization for chlamydial diagnostics may be arrived at.

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