
INCIDENCE OF PIG CHLAMYDIOSIS IN LITHUANIA REVEALED BY DIFFERENT TECHNIQUES

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ABSTRACT

Pig chlamydiosis is antrophozoonosis due to Chlamydophila abortus. Chlamydias (C type) are widely spread in nature. This infection could be in humans, domestic and wild animals and 139 types of birds (3, 7, 13, 14). The peculiar feature of chlamydias is the tissue tropism of different organs and organisms. They cause various diseases of the acute and latent development. Very often it is asymptomatic.

In 2502 pig blood sera tests from Lithuanian farms anti-chlamydia complement binding (CB) antibodies were detected in 192 cases (7.7%). Serological tests show the following (C type) chlamydia bearing regions: 22.0% Mazeikiai district (2.53 ± 1.06), 17.2% - Kaisiadorys district (2.57 ± 0.63), 13.5% - Panevezys district (1.88 ± 1.04), 12.3% - Vilkaviskis district (1.98 ± 1.5). Rare incidence of the disease was found in Siauliai district 1,2% (1.58 ± 0.00) and Klaipeda district 2.5% (1.58 ± 0.00) farms. The highest antibody titers in blood serum test were found in Joint Stock Company (JSC) "Krekenava" and "Vejine", i.e. 1:128 and 1:64 (1.44 ± 1.77), respectively.

The lowest levels of these antibodies (1:8) were registered in JSC „Vycia” (1.00 ± 00) and Agricultural Company "Verbunai" (1.58 ± 0.00).

In pigs group with the clinically expressed symptoms 108 pigs infected with chlamydia were detected. Antibody distribution by CB assay, in piglets, sows and boars showed the infection rate from 3.4% to 7.9%. In sows with clinical symptoms the incidence of chlamydiosis was 3.0% greater in comparison with all sows investigated. In piglet the positive reaction of serum was 5 times less. The highest level of Chlamydia infection was in fattening pig group (17.6%).

Seroepizootic study of pig chlamydiosis indicates the different infection rate in the animals investigated. The biggest number of infected pigs was in 1990 (28.7%), then it gradually decreased and in 1995 reached only 10.1% (or decreased by 18.3%). The disease is also season-dependent. The biggest infection risk is in winter (10.4%) and lowest - in summer (2.8%).

The following methods for the study of pig chlamydiosis were used and comparatively evaluated: complement binding reaction (CBR), direct immunofluorescence (DIF), immunoenzyme assay (IEA) indirect immunofluorescence (IIF), micro immunofluorescence (MIF), polymerase chain reaction (PCR) and cell culture (CC) test. PCR method was found to be more sensitive and reliable than that of immunoenzyme assay, but the latter is more economic especially for screening.

Introduction

Pig chlamydiosis is antrophozoonosis due to *Chlamydophila abortus*. The disease is widely spread and can be detected in humans, animals, beasts and 139 strains of birds (1, 3, 7, 13, 14). The specific feature of chlamydiosis is the tissue tropism of different organs and organisms. Therefore the World Health Organization confirmed the programme for investigation of biological properties of Chlamydias and improvement of diagnostical methods. Chlamydias can cause the acute and latent disease forms. Very often the disease is asymptomatic.

By the current methods of Chlamydia genome restriction and molecular hybridization the primary gene structures 16S and 23S rRNA acquired additional phylogenetic traits for different Chlamydiales. The study of gene systematization tests were of great importance for the classification of chlamydias: according to a new taxonomy Chlamydia strain from Chlamydiaceae family with *C.*

trachomatis, *C. suis* and *C. muridarum* was defined (**Fig. 1**). To this *Chlamydophila* strain *C. pneumoniae*, *C. pecorum*, *C. psittaci*, *C. abortus*, *C. caviae* and *C. felis* types can be included. The representatives of both strains are isolated from different hosts and they are different according to biochemical, antigenic properties and their virulence degree (12). The current changes in the classification of chlamydias show the developing process in their study as biological object. For the first time pig chlamydiosis was described in 1959 by V. Sorodok, Romania, J. Guenov 1961 Bulgaria, B. Natscheff 1965 Hungary, M.R., V.A. Bortnichuk (1991), the former USSR. C. Prudnikov in 1963 - 1964 in Romania investigated the outbreak of pig abortion and loss of one week sucking pigs. There was a case of 38% of pig abortion and a loss of 7% of sucking pigs on one farm. According to the investigators the clearly expressed tropism in sow genital epithel cells was detected. In 1968 F. Blanco in Spain

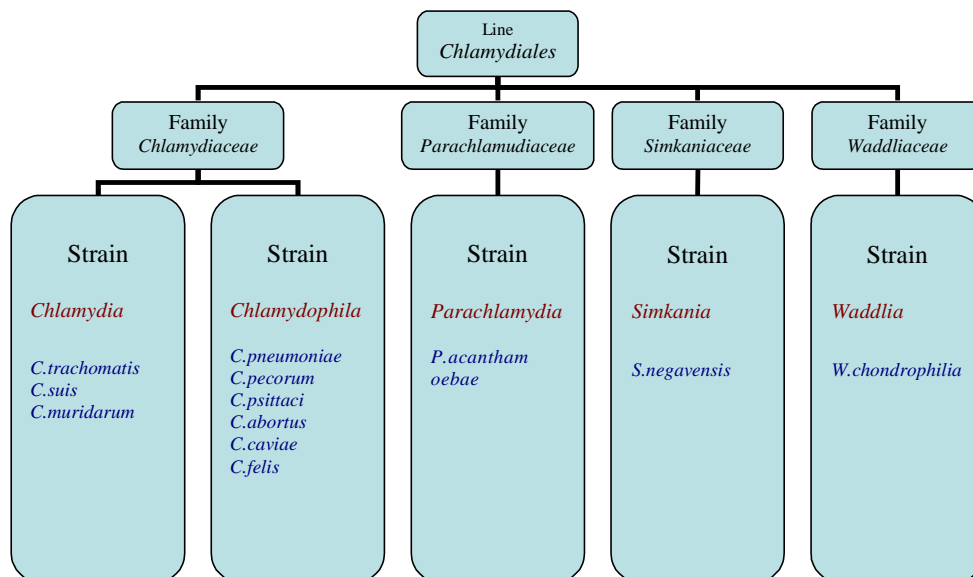


Fig. 1. Classification of Chlamydias.

described sporadic cases of pig abortion and loss of piglets, due to chlamydiosis. In Russia N.F. Shcherban et al. (1982), stated, that chlamydias in pigs can cause not only bronchopneumonia, but also urogenital pathology. The authors succeeded in performing artificial abortion in pregnant sows and induction of pig chlamydiosis. During experiment 58.1% of piglets were stillborn and 6.6% expressed only low viability. V.I. Kovoliov in 1975 described the case on a farm where 130 pregnant sows had an abortion and more than 3000 two and a half month old piglets were ill with bronchopneumonia. Some boars were diagnosed to have urethritis, orchitis and sterility. The inflammation of urogenital organs and joints was detected. The outbreak of chlamydiosis could be due to formation of pig farms with the animals from different location. The immunological status and microbiological background is effected due to stress situation (2, 3).

Pig chlamydiosis is in the focus of investigators because up till now the pathogenicity of the pathogen for humans is not clear. In 1985 pig chlamydiosis was started to be investigated in Lithuania (1), although the incidence of the disease, epidemiology, the influence of the inducer for respiratory and genital diseases, the comparison and evaluation of diagnostical methods and prevention means was not completely performed. While the international economical and veterinary communication is developing more cases of mixed infections, chlamydiosis including, are found. Therefore it is of importance to investigate the incidence of pig chlamydiosis in Lithuanian farms, to compare the diagnostic methods used, to prepare and evaluate new diagnostic techniques.

Materials and Methods

Samples from small private farms and 10 pig breeding companies were tested to eval-

uate the epizootic situation of pig chlamydiosis. The work was effected in 1990-2002. Detecting animals with clinical symptoms, the pathological material and blood samples were investigated. The swabs, mucous and pathological material was sampled from piglets, boars and sows. 2502 samples were collected. To prepare cell cultures 8-9 day old chicken embryos were used to cultivate chlamydia. The immune serum and immunoglobulin (Ig) was obtained by immunization of 4-5 kg weight healthy rabbits (into ear vein).

All investigations were performed in the compliance with the laws of Lithuania for "Animal Protection, Welfare and Use", ("State News No. 108, 28/11/1997) and with by-laws of Lithuanian State Food and Veterinary Service "The Veterinary Requirements for Breeding and Keeping of Laboratory Animals their Welfare and Transportation" (31/12/1998, No. 4-361) and "The Use of Laboratory Animals in the Scientific Investigations" (19/01/1999, No.4-16).

As a reference Chlamydia strains isolated from bull semen (N9) and a standard one isolated from parrot (LO-7) was used. Specific and control antigens were prepared according to the method of Volkert M. and Christensen P. 1955, modified in our laboratory. Antigen activity and quality was tested by CBR using immune and control sera from D.F. Ivanovskij Institute of Virusology (Moscow) and commercial products from Odessa and Cherson biofactories. Antigenic properties were investigated by CBR, using a standard ornithose antigen and inactivated yolk sack suspensions. Special sterile media for specimen transportation (Syva Mikrotract, USA) or cell culture growing medium was used. After that streptomycine (100-200µg/ml), kanamycin (100µg/ml) or gentomycin (2µg/ml) was added. Small pieces of internal organs (lungs, liver, spleen,

lymph nodes, intestines, brain and affected joints) were sampled from dead animals. In case of abortion the pieces of parenchymal organs of embryo, secretes from vagina and swabs from vagina walls and placenta were sampled. Swabs were fixed by cold methyl alcohol and kept at the temperature of 2-8°C. Samples from eye mucous membrane, cervical and preputium, were studied by CC technique and DIF method. Swabs were put into thawed specific transportation medium for PCR, (LCx STD Swab Specimen Collection System, Abbot laboratories, USA). CC tests were performed according H.D. Isenberg 1922. Chlamydiosis was diagnosed by clinical symptoms, pathology and laboratory data from the farms with higher risk. By light and luminescent microscopy chlamydias were studied in tissue of affected organs. The diagnosis was confirmed by CC, PCR, CBR and ICBR according to 50% haemolysis of erythrocytes. Sera were diluted from 1:4 to 1:128. The test was done according to the technique by I.I. Terskich (14). In DIF „Chlamifluran” (Russia) and „Kit Imogen IF test” (France) was used. IIF was effected with the rodomine labeled pig serum albumin and species specific anti IgG labeled by fluoresceinizotiocyanate. MIF was done as per C. Javetz (1970). Inactivated Chlamydia psittaci antigen produced in the N.F. Gamalea Institute of Epidemiology and Microbiology was used. FITC labeled mouse anti-pig IgG antibodies (by „Orion” company) were used. On the plate with the material investigated 5µl of antigen was added and fixed for 10 minutes in frozen absolute ethanol (C₂H₅OH-99,5%). Later sera for investigation were added in the dilutions from 1:8 to 1:1024. The contrast was obtained by bovine albumin, labeled by Evans Blue or rhodamine. IFA performed according to M. Domeika et al. (18). The antigen from Chlamydia strain LO-7 was used. An-

tigen was diluted 1:200; and initial sera diluted by 1:10. The results were evaluated by microspectrophotometer “Sumal” and “Syva Mikrotrac” PCR was done as recommended by D.R. Pollard (9). For the test was used DNA short nucleotide sequences, named C. psittaci (CMOMPM / CPSMOMPC), C. trachomatis (CMOMPN / CTMOMPC) and C. pneumoniae (CMOMPN / CPNMOMPC). To detect chlamydias in CC, chicken embryos and McCoy cells were infected. They were cultivated in medium 199, enriched by 10% of bovine embryo serum. Cells were removed from glass surface by 0.25% trypsin solution, produced at the Lithuanian Veterinary Institute. Cells were pretreated by 90 µg/ml of diethylaminoethyl – dextrane DEAE-D (MW. 2000000, Pharmacia, Switzerland). McCoy cells were treated by 1.0-0.9 µg/ml Cychlohexamide (Koch-Light, England). Infected cells were tested in DIF using “Kit Imagen Chlamydia”, “Chlamyset” diagnostic sets. The results were evaluated (screened) with luminescent “Nycon” Microscope (6).

The data was evaluated by the methods of mathematical statistics. Arithmetical mean (M) was calculated and deviation allowed (±m). The data are considered to be reliable, when p<0.05. The graphical presentation and statistical parameters were processed by Microsoft Excel 7.0 programm.

Results and Discussion

Pig blood samples were taken from 24 farms. Two farms were antichlamydia antibody negative. In three districts from 0.1 to 5% of samples were positive. In six districts from 5-10% of pigs were positive. The highest incidence 20-25% of the disease was found in Mazeikiai, Kaisiadorys and Panevezys districts. If to look at the map of Lithuania, the right side of the river Nemunas is seen as high risk region. After testing

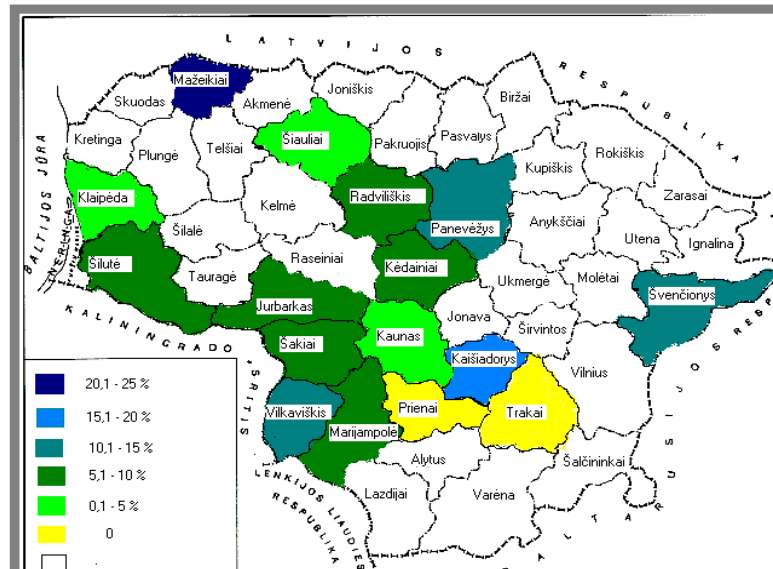


Fig.2. The Serological Data on Pig Chlamydia=

of 2502 pig blood sera, antichlamydia CB antibodies were found in 192 samples (7.7%). Serological tests show 22% (2.53 ± 1.06) of chlamydia infected pigs in Mazeikiai district, 17.2% (2.57 ± 0.63) in Kaisiadorys district, 13.5% (1.88 ± 1.04) in Panevezys district, 12.3% (1.98 ± 1.5) in Vilkauskis district. In Siauliai and Klaipeda districts the number of infected pigs is the smallest 1.2% (1.58 ± 0.00) and 2.5% (1.58 ± 0.00), respectively see **Table.1**.

More detailed analysis of separate farms shows, that antichlamydia antibodies were detected in JSC "Krekenava" 13.8% (1.83 ± 0.84) and in the Agricultural Company "Zibartony" – 12.9% (1.93 ± 1.25). The highest rate of CB antibodies was detected in JSC "Krekenava" and JSC "Veji", i.e. 1:128 and 1:64 (1.44 ± 1.77), respectively. The lowest amount (1:8) of the antibodies was found in JSC "Vycia" (1.00 ± 0.00) and in Agricultural Company "Virbunai" (1.58 ± 0.00).

Serological data show that from 24 farms investigated 7.7% of pigs are infected. The most noticeable divergence from the average infection rate is in Mazeikiai (14.3%) and Kaisiadorys district (9.5%). CBR test results of pig blood samples from JSC "Zelve" and farms from Prienai district were negative. Analyzing samples from JSC "Krekenava" 7.4% of sera were positive at the dilution 1:8. In Mazeikiai district this number reached 14.7%, in Kaisiadorys – 8.6%. Results show, that in 4.1% of pig blood sera antichlamydia CB antibodies were found at the dilution 1:16. However in Mazeikiai district this percentage reached 14.7% using the same dilution. In the JSC "Balciunai" 3.4% (1.98 ± 1.5) of pig blood sera were chlamydia antigen positive at the dilution 1:32 and 1.4% - 1:64. Quantitative analysis of antichlamydia antibodies shows, that 4.4% of pig blood sera were positive at 1:8 and at least 0.1% at 1:128 dilutions.

Table 2 depicts the classification of the 2502

TABLE 1

The Incidence of Pig Chlamydiosis

No.	District	Serum investigation	Positive	%	log ₂ M±m
1.	Jurbarkas	107	10	9,3	2.20±0.86
2.	Kaišiadorys	116	20	17,2	2.57±0.63
3.	Kaunas	602	22	3,6	1.23±0.44
4.	Kedainiai	19	1	5,3	0.00±0.00
5.	Klaipeda	40	1	2,5	0.00±0.00
6.	Marijampole	308	16	5,2	1.09±1.14
7.	Mazeikiai	109	24	22,0	2.53±1.06
8.	Panevezys	349	47	13,5	1.88±1.04
9.	Prienai	22	0	0	0.00±0.00
10.	Radviliskis	77	6	7,8	1.58±0.00
11.	Sakiai	56	3	5,4	0.50±0.71
12.	Siauliai	241	3	1,2	1.58±0.00
13.	Silute	117	6	5,1	1.29±0.41
14.	Svencionys	141	15	10,6	1.44±1.77
15.	Trakai	52	0	0	0.00±0.00
16.	Vilkaviskis	146	18	12,3	1.98±1.5
	TOTAL	2502	192	7,7	1.24±0.59

samples tested according to animal groups: i.e. pigs, gilts, sows and boars. In the group of ill pigs 983 samples were tested. 108 of them were found to be chlamydia positive. Serological data show, that CB antibody distribution in different pig groups reveals the infection rate from 3.4% to 7.9%. However, in ill pig group it makes 17.6%. Fattening pigs were infected with chlamydia 14.2% more than piglets. The samples from ill sows showed the greater incidence of chlamydiosis in comparison with all sows investigated. CB study revealed the correlation between the disease and the age of animals, i.e. the antibodies were found in 3.4% of piglets, in 8.8% of boars in 10.8% of sows. Samples from piglets were 5 times less positive (3.4%). The group of fattening pigs consisted of pigs unfit for reproduction, hav-

ing been ill with different diseases (pneumonia, enteritis, metabolism disorders etc.). Boars were infected very similarly as sows 18.8-10.8%, respectively.

Serological tests were performed in 1990-2002. The number of samples tested per year was different, e.g. 27 samples in 2001 and 719 samples in 1996 (**Fig. 3**).

Comparing our data with those obtained by V.A. Bortniciuk (17) it could be noted that the incidence of chlamydiosis in Lithuania was 4.1 times lower. 31.6% of positive samples were found in Ukraine, as in Lithuania this percentage was only 7.7. Antibody titres show the latent form of the disease. In abortive animals antibody titres were 1:10, 1:40 (ICBR). Our study shows the dependence of chlamydia positive animals on the farm. Some authors think, that it may de-

TABLE 2

Serological data According to Animal Groups

Animal group	Samples Number of samples investigated	Total		III		
		positive.	%	Number of samples.	positive	%
Piglets	59	2	3.4	59	2	3.4
Fattening pigs	432	21	7.6	108	19	17.6
Sows	1935	94	7.8	748	81	10.8
Boars	76	4	7.9	68	6	8.8
Total	2502	192	7.7	983	108	11.0

pend on the seroepizootic situation of the farm, the season of the year and the age of animals (Chamadeev N. A. et al., 1990; Pospischil A. et al., 1996). The data show that in the sick animals group the infection rate is even 6.1% greater in comparison with the total number investigated. Older pigs are 6.4% more infected than the younger ones. Seroepizootic analysis shows the diversity of the infection (Fig. 3). The worst year for

pig chlamydiosis was 1990 (28.7%). Then it gradually decreased and in 1995 only 10.1% of disease cases were registered (decreased by 18.3%). Although in the late nineties, the number of pigs ill with chlamydiosis started slightly to grow and in 1999 the percentage was 5.1% higher than four years ago. Current studies show the decrease of the disease again due to better keeping conditions and means of prevention (4-8%).

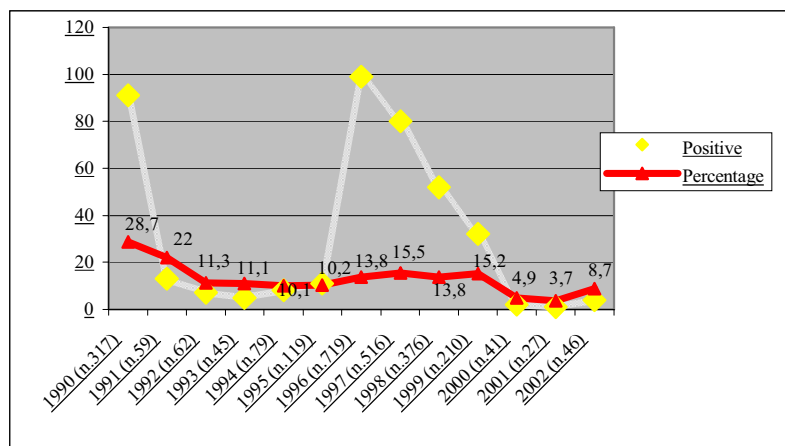


Fig. 3. Investigations of Pig Chlamydiosis by ICBR and CBR in 1990-2002.

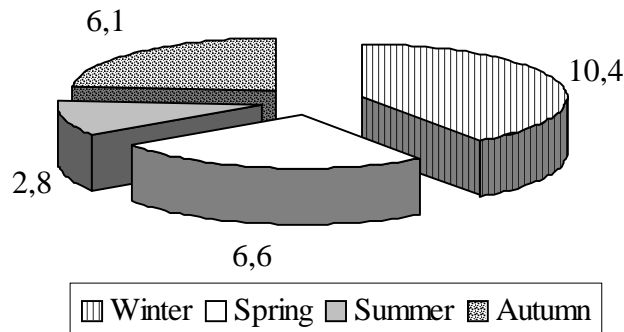


Fig. 4. Season – Dependence of Pig Chlamydiosis (1990-2002).

Season – dependence studies show the different infection rate during the year. The highest infection level was found in winter. CB antichlamydia antibodies were detected in 10.3 % samples of pig blood serum. The lowest infection rate was found in summer samples (i.e. 9.4% less) to compare with the cold season of the year. The data in spring and autumn differ insignificantly 6.6 and 6.1%, respectively. When the comparison was made according to different months, the highest incidence rate was in December and February, the lowest – in August and June (0% and 2.8%), respectively. Our data is in correlation with the studies of V.A. Bortniciuk (17). They found that the highest incidence of the disease was in February – April. A slight increase could be noticed in June, due to the presence of pathogen. This increase could be called “false season prevalence” and is repeating every 3-4 year.

The main symptom to diagnose pig chlamydiosis on a farm is pig abortion and still born piglets. Epizootic abortion of sows (30-40%) usually occurs during the first farrowing. The embryo is injured first though the symptoms of infection before the farrowing and after it remain unnoticed (4). Our data show that from 39 aborted pigs with or without farrowing of dead piglets, 46.1% were

chlamydia positive. From 61 pigs with vaginitis tested, 22.9% were ill with chlamydiosis. In aborted embryos, hypodermic bleeding in the area of thymus, nasal, pharynx, trachea, in the mucous membranes of gullet and muscular tissues were found. In the cavity of stomach, thorax and in the heart membrane, blood bearing or light colour exudates could be detected. Liver is enlarged, breaking with small nodes. Spleen and lymph nodes are enlarged too. In many organs and tissues gray areas of 5-10 mm are detected (2, 20).

Chlamydiosis is mostly of chronic disease type. Infected animals discharge the pathogen with faeces, though the clinical symptoms of the disease are absent. C.Rothermel et al. (11) states that the natural medium for chlamydia is the alimentary tract. The latent presence of chlamydia in the alimentary tract could cause the systemic infection of the organism, when chlamydia reach the epithelial and cellular layers. When the lymph system is affected the process is called chlamydiosis. In 4.6% from 625 pigs tested chlamydiosis was detected though clinical symptoms were not found or they bore a very weak expression. 29 pigs of different age were ill with chlamydiosis of asymptomatic type. M.K. Stefan et al. (22) confirms that

TABLE 3

The Comparison of Methods Used to Define the Antibodies for Pig Chlamydiosis

Number of samples from separate farms	CBR	%	DIF	%	IEA	%	IF	%	MIF	%	p
1633	259	15,8	487	26,1	-	-	-	-	-	-	<0,01
614	85	13,8	-	-	219	35,7	-	-	-	-	<0,001
143	23	16,1	-	-	-	-	41	28,6	-	-	<0,05
94	19	20,2	-	-	-	-	-	-	30	31,9	>0,05

in chlamydia abortion negative farms there is the reverse correlation between the serologically positive results and the incidence of the disease.

Serological tests used to diagnose pig chlamydiosis (ICBR, CBR, IFM) are sensitive and reliable, though a single test of pig blood serum does not allow to diagnose chlamydiosis.

Some authors state, that good correlation of the results could be obtained by immunofluorescence test and chlamydia isolation in chicken embryos. Correlation is proved in 70-80% (21).

The comparison of the methods used allows to state, that using IEA 42.2% of specific antibody bearing pigs was diagnosed it is 3.4 times more than using CBR.

By DIF technique 1633 samples were tested, 487 positive cases were found. It is 1.6 times more than by CBR. It shows that DIF is sensitive and specific: using IIF 143 samples were investigated. Pathogen positive cases were found 1.8 times more than by CBR. 94 specimens studied by MIF showed 1.6 times more ill animals, than by CBR. MIF in our studies was 11.7% more sensitive than CBR. MIF is also *C. psittaci* strain specific showing the increased amounts of IgG and IgM. IgM generally disappears after 2-6 months from the outset of the infection, but IgG could be found for several years. MIF test gives evidence about the process of the disease. This method is not used up till now in Lithuania for Chlamydia testing in animals.

CBR and DIF were performed collecting 1633 samples from 7 farms (Table 5). By CBR antichlamydia antibodies were detected in 259 (15.8%) samples of blood serum. With the help of DIF 487 (26.1%) cases were registered. It is 1.6 more than by CBR ($p < 0.01$). IEA was used to test 614 blood samples. 35.7% of pig chlamydiosis was di-

agnosed. It is 2.6 times more than by CBR (13.8%; $p < 0.001$). 143 specimen collected from 3 separate farms were tested by IIF – 28.6% were found to be Chlamydia positive or it is 1.8 times more, than showed by CBR (16.1%; $p < 0.05$). Analysis of 94 samples by MIF and CBR confirmed 31.9% positive samples and 20.2%, respectively, i.e. the first technique revealed pathogenicity 1.6 times more than the second ($p > 0.05$)

The comparison of methods for pig genital chlamydia shows, that the biggest number of chlamydia positive cases (35.7%) was defined by IEA, which was 2.6 more sensitive than CBR. MIF technique was 11.7% more sensitive than CBR. 252 samples collected from 2 farms were tested by DIF and CC. The comparison of these two methods revealed, that CC is 1.6 times more sensitive than DIF. According to their specificity and sensitivity CC and DIF are inferior only to PCR (14.3% and 22.9 %), respectively. According to our earlier data we can calculate that PCR is very sensitive and reliable method to diagnose chlamydia.

For PCR tests the material from 83 ill pigs was collected. 36.6% of chlamydia – positive cases were found. It is 22.9% more than by DIF. While studying 252 samples by DIF and CC, we detected that CC was found to be 8.4% more sensitive than DIF. PCR can also be applied to differentiate between the separate strains of specific 1200 base length DNA fragment (CMOMPM / CPSMOMPC) from *C. trachomatis* (CMOMPN / CTMOMPC) and *C. pneumoniae* (CMOMPN / CPNMOMPC). The comparison of DIF, PCR and CC methods is the following: PCR is 24 times more sensitive than DIF ($p < 0.05$) CC is ($p < 0.01$). PCR is 1.6 times more exact than CC.

Our data is in correlation with the comparative analysis of the methods done by other authors. The most sensitive and reliable is

PCR technique by which we can define 38.5% of pigs bearing *C. psittaci* DNA. To have a general view on the incidence of chlamydia DIF, PCR and CC can be used. They are more economic and faster than isolation of using chicken embryo and/or laboratory animals. To diagnose chlamydia it is not enough to perform serological, epidemiological and pathomorphological tests. The presence of chlamydia should be confirmed by DIF, CC and/or PCR techniques.

Conclusions

1. Serological tests showed the incidence of pig chlamydia in 87.5% of districts in Lithuania. Antichlamydia bearing antibody quantitative evaluation was $1.24 \pm 0.59 \log_2$ that shows the chronic type of the disease. In 2502 pig blood serum tested, 7.7% were antichlamydia CB antibody.
2. The incidence of pig chlamydia is season-dependent and connected with the age of the animals. The majority of the disease cases are found in winter (10.3%), the lowest – in summer (0.09%). The biggest number in the fattening pig group (17.6%) and the lowest in piglets (3.4%).
3. In the studies of clinical material from different animal groups by the method of DIF 26.1% samples were specified to be chlamydia antigen positive.
4. By PCR technique 38.5% of pigs bearing *C. psittaci* – the inducer of chlamydia was detected. The results show that PCR is 1.6 times more sensitive than CC and allows to differentiate strains *C. psittaci*, *C. trachomatis* and *C. pneumoniae* in different biological samples.

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