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Research Article

Studies on Porcine Circovirus Infections in Bulgaria

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Abstract

Circovirus infections are a current and significant veterinary and economic problem in a number of countries with developed pig breeding. Clinical manifestations of the disease caused by Porcine Circovirus Type 2 (PCV 2) include Postweaning Multisystemic Wasting Syndrome (PMWS), Porcine Dermatitis and Nephropathy Syndrome (PDNS), respiratory, reproductive or intestinal disorders, subclinical infections (PCV 2 SI). Circovirus Type 3 (PCV 3) is responsible for cardiac and multisystemic infections, PDNS, febrile conditions, pneumonia and reproductive failure. In the present study are presented data for etiology, the spread of infection among the populations and categories of pigs in Bulgaria and its clinical manifestations and histopathology, as well as the applied modern approaches for diagnostics and research. Employed were serological methods (indirect ELISA with recombinant antigen and monoclonal antibodies), immunohistochemistry (IHC), in situ hybridization (ISH), PCR techniques, sequencing of isolates. High seropositivity was found among different categories pigs (domestic, Eastern Balkan breed, wild), which shows a wide prevalence of PCV 2 infection among pigs in the country. Most susceptible group was adolescent pigs at 12-14 weeks of age. The incidence varied from 30 to 60% and mortality - from 3 to 20%. IHC detected viral antigen in lymph nodes, spleen, tonsils and lungs. The application of

ISH method is effective. By conventional PCR of blood samples from pigs were obtained DNA amplicons of 656 bp and in the study of specimens from lymph nodes - amplification products with size of 494 bp were found. By sequencing the PCV 2 isolates were received data for difference in nucleotide and amino acid sequences of the capsid gene of Bulgarian isolates and reference strains. The Bulgarian PCV 2 isolates Han Asparuh 4 and 19 belong to PCV 2b genotype and represent a separate branch from the referent isolates. Te third Bulgarian PCV 2b isolate Rousse also is separate branch based on its differences with the mentioned Bulgarian PCV 2 isolates, as well as with reference isolates from Spain, Netherlands and Scotland. Most typical histological changes for circovirus infections were found in lymph nodes characterized by different degrees of granulomatous inflammation. Frequent findings in the lungs were purulent, interstitial or fibrinous pneumonia.

Introduction

Significant prevalence of porcine circovirus infections in recent years throughout the world has led to major economic losses for pork producing countries. Porcine Circovirus Type 2 (PCV 2) is considered the major cause of a number of pig diseases such as post weaning Multi systemic Wasting Syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), reproductive disease, enteritis, respiratory distress, acute pulmonary edema, subclinical infections. A new member of the Circoviridae family has been found in recent years - the porcine circovirus 3 (PCV 3) [1,2]. The Infections with PCV 3 cause cardiac and multi systemic inflammation in pigs [2], PDNS, reproductive failure in sows [1,3] as well as fever and pneumonia in pigs [4]. Despite considerable progress, a number of circovirus-related issues remain unclear, contradictory and insufficiently explored. Here are the speculative elements in epidemiology, the assessment of the veterinary and economic significance of subclinical infections, the mechanisms allowing the expression of disease and immunity, a better definition of risk factors, concurrent infections and coinfections with other agents, etc.

All this requires ongoing research efforts on a broad experimental basis.

With this study we aim to investigate certain aspects of circovirus infection in pigs in Bulgaria: distribution, advanced diagnostic approaches, clinical scope, pathology.

Material and Methods

Serology. We examined 438 blood samples from domestic pigs from farms or private yards, pigs from Eastern Balkan breed and wild boars from a total of 14 regions of the country. We used a diagnostic kit Ingezim Circo IgG 11. PCV. K.1 based on indirect ELISA including recombinant antigen (VP 2 protein of PSV 2) and monoclonal antibody (Mcab) specific for porcine immunoglobulins (IgG).

Immunohistochemistry Test (IHC) To Prove the Antigen of PCV Type 2. Samples of lymph nodes, tonsils and lungs from 89 pigs were examined. We applied an indirect two-step variant of IHC using secondary biotinylated antibodies and those with enzymes and polymers attached to them [5].

In Situ Hybridization (ISH) for the Detection of PCV 2 DNA. We examined lymph nodes, tonsils, lungs and small intestines from 60 animals. The reaction was carried out according to a protocol described by Rosell *et al.* [5].

Molecular Biological Studies

Conventional PCR to Prove PCV 2 DNA in Blood Samples. We tested a total of 190 blood samples, 128 of which were from a pig farm and covered all age groups. To amplify the DNA obtained from the samples, we used primers with the following nucleotide sequence:

Forward: 5' CACGGATATTGTAGTTCCTGGT 3,
Reverse: 5, CCGCACCTTCGGATATACTGTC 3,

Conventional PCR to Prove PCV 2 DNA in Tissue Samples. To amplify the genetic material primers multiplying ORF 2 PCV 2 region, size 494, were used:

Forward: 5, CACGGATATTGTAGTCCTGGT 3,
Reverse: 5, CCGCACCTTCGGATATACTCTC 3,

Real-time Polymerase Chain Reaction (Taqman PCV 2) for Detection of PCV 2 DNA in Tissue Samples.

We used primers and probes as follows:

PCV 2 F: CCAGGAGGGCGTTGTGACT (1535-1553);
PCV 2 R: CGTTACCGTTTGGAGAAGGAA (1633-1614)

PCV 2 P: FAM-AATGGCATCTTCAACACCCGCCTCT-TAMRA (1612-1592)

Sequencing of Isolates to Prove PCV -2. To establish the genotype of circulating PCV 2 strains in Bulgaria, we used blood samples and tissue samples positive for PCV 2 DNA in PCR. A total of 100 samples were tested: 76 blood samples and 34 suspensions of lymph nodes, lung, and skin. Control positive samples: 2638 Lelystad NL; viral isolate 1010 Scotland; a positive DNA sample of ELISA kit Ingezim Circo IgG 11. PCV. K.1. We have sequenced 6 isolates - 3 Bulgarian and the above three. The resulting sequence products of ORF 2 gene were compared with known gene sequences from reference isolates stored in the gene bank. To build a phylogenetic tree of the isolates we used MUSCLE algorithm [6] and MRGA 7 software [7] and 32 sequences of strains deposited in the NCBI, USA.

Clinical Studies. These observations performed in 23 pig farms from different parts of Bulgaria using the criteria of Opriessnig *et al*., 2007 [8] and Segales *et al*., 2008 [9] to determine the type of infection with PCV 2.

Pathological Anatomy and Histopathology. Pathologically, we surveyed 222 dead or slaughtered pigs from the above 23 pig farms. The autopsies were performed according to the method described by Stoev *et al.* 2016 [10]. The histopathological studies covered 112 animals aged 12 to 150 days.

Results and Discussion

Clinical Observations

In all 23 pig farms with a total number of 222 animals - adolescents and fattening, we have identified clinical signs of PCV 2 systemic disease (PCV-2-SD), formerly known as PMWS. In addition, in 12 of the above holdings we also observed pigs (12 / 12.7%) with necrotic skin changes characteristic of Porcine Dermatitis and Nephropathy Syndrome (PDNS). We also found cases of PCV 2 subclinical infection (PCV 2-SI).

Most susceptible to infection are adolescent pigs aged 12-14 weeks. The incidence of PCV 2 infection usually reached 30%, and much less - 50-60%. Mortality in all farms was increased and ranged from 3 to 20%.

Serology

These data are presented in Table 1. It shows that in the study of 437 pigs from three categories, coming from 33 farms and hunting areas in 14 districts, seropositive for infection with PCV 2 were 366 (83.8%) respectively 94.4% - domestic swine, 79.2% - Eastern Balkan pig and 65.35% - wild boars. The rates of seropositive reactions are high in all three categories of pigs, which is likely to serve as an indicator of contacts between them.

Table 1: *Serological data of PCV 2 distribution in pigs from three categories, districts and number of tested animals.*

Categories of pigs	District	Number of farms	Tested animals [number]	Positive [number/%]
Domestic swine	Blagoevgrad	2	20	20/100
	Vratsa	1	5	5/100
	Gabrovo	5	58	58/100
	Kyustendil	1	12	12/100
	Montana	2	12	12/100
	Pleven	1	12	12/100
	Razgrad	2	32	29/90
	Rousse	2	16	8/50
	Yambol	1	10	10/100
Total	9	17	177	167/94.9
Eastern Balkan Pig	Burgas	4	72	46/63.9
	Varna	2	32	32/100
	Shoumen	5	107	89/83.2
Total	3	11	211	167/79.2

Wild Boar	Blagoevgrad	4	40	27/67.5
	Kardzjali	1	9	5/55.6
Total	2	5	49	32/65.3
Total	14	33	437	366/83.8

These data indicate a widespread prevalence of infection among the pig population throughout Bulgaria. Reports of widespread dissemination of PCV 2 in different parts of the world were presented by a number of authors [11-17].

Immunohistochemistry

For these studies, samples of pigs aged 59-126 days with lymph node changes were selected. Positive results for infection with PCV type 2 were obtained in 11 cases - 12.4%. With the use of two variants of the IHC, the results are similar, but more sensitive and faster is the response comprising a polymer linked to a peroxidase and a secondary antibody. This reduces the duration of IHC, reduces the likelihood of non-specific reactions and gives a more intense coloration. Viral antigen was detected in the lymph nodes, the spleen and the tonsils mainly in the B-cells areas of the lymphoid organs, as well in the lungs, liver and kidneys We observed a typical brown color labeling of the viral antigen, mainly in the cytoplasm and, more rarely, in the nucleus. The Type 2 virus antigen is localized diffusively in the cytoplasm of large cells with macrophage morphology, in multinucleated giant cells, and to a lesser extent in epithelial cells and also in multiple polymorphic intracytoplasmic inclusions in histiocytes. In swine lymph node samples, we found an intensively stained viral antigen in the cytoplasm of histiocytes and dispersed around the follicular areas (Figure 1A). A well-pronounced histochemical reaction was also observed in the cytoplasm of lymphocyte nuclei and large macrophages as well as in interstitial tissue of the tonsils (Figure 1B). Through the IHC method, we have been able to confirm the presence of PCV 2 in the lungs. The antigen was detected in the large macrophages of the affected alveoli and in the cell debris in the bronchioles (Figure 2).

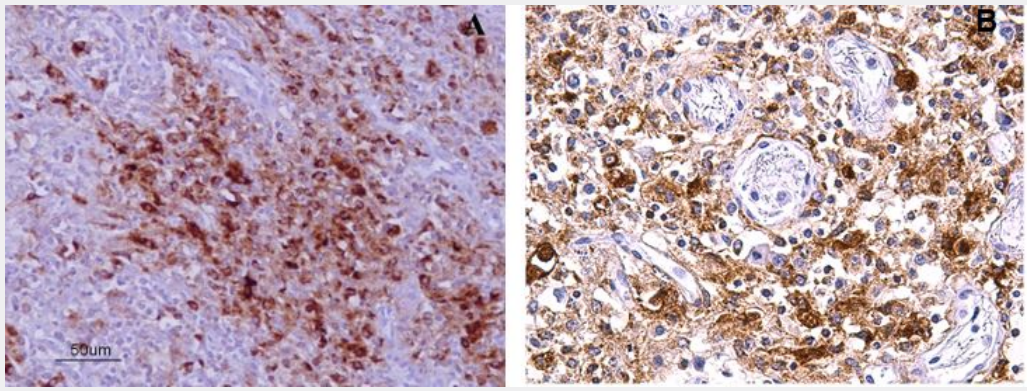

Figure 1: *Lymph node (A). Intensive diffuse labeling in the cytoplasm of macrophages in 85 days age pig. Tonsil (B). Nuclear and cytoplasmic labeling in the mononuclear cells in lymphoid follicles in 80 days of age pig.*

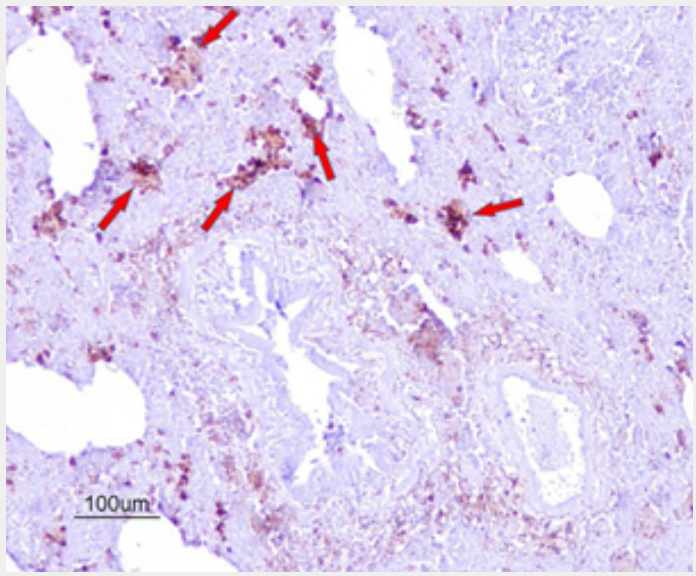

Figure 2: *Lung. Positive IHC labelling for PCV 2 antigen distributed in affected alveoles and lung parenchyma (arrows) in 81 days of age pig with PCV 2 SD.*

In Situ Hybridization

For performing ISH used labeled complementary strand of DNA in order to localize the specific PCV 2 DNA in tissues. Positive results were found in 11 (18.3%) of the tested animals. The nucleic acid of the virus has been demonstrated in lymphoid organs - lymph nodes and tonsils, as well as in the lungs and small intestine (Figures 3, 4). An intense ISH reaction to the presence of PCV 2 DNA has been detected in the cytoplasm of macrophages, follicular dendritic cells and giant cells in the lymph nodes. Intensive specific signal for PCV 2 DNA is most often found in the cells of germinal centers (Figure 3 arrows). In the lungs the intensity of ISH was weaker. Viral DNA is found in the cytoplasm and nucleus of mononuclear cells infiltrating alveolar lumen and septa and also the peribronchial cells of the lung tissue (Figure 4 arrows). Intensive staining of PCV 2 found in the mononuclear inflammatory infiltrate and in the epithelial cells of the small intestine mucosa. The presence of viral DNA detected by ISH indicates that PCV 2 replication is performed in the lymphoid cells. The sensitivity of ISH was confirmed by RT-PCR of 10 positive lymph node samples (ISH). And the ten samples were positive in RT-PCR. In our studies we used specific sequences from the genome of PCV 2 with which the specificity and sensitivity of the reaction was very high. Overall, we found a higher sensitivity of ISH than that of IHC. This may be due to the greater amount of DNA in the tissues compared to the proteins proven by IHC.

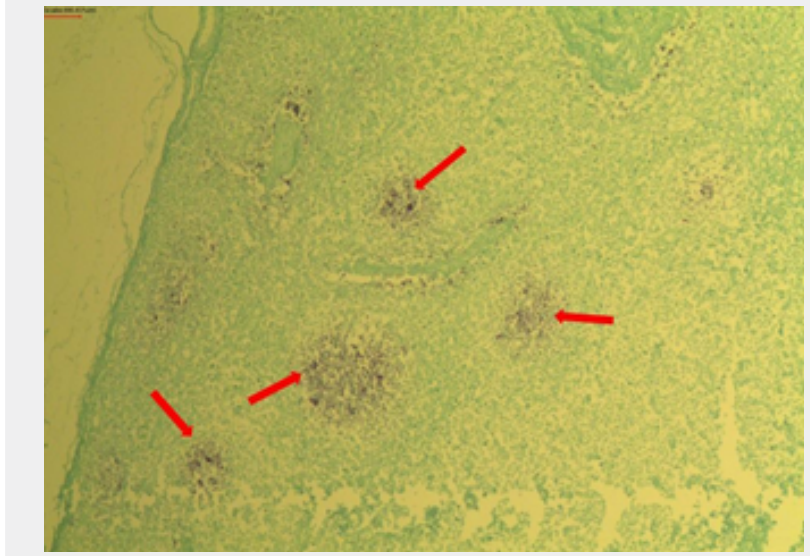

Figure 3: *Lymph node. PCV 2 nucleic acid detected by ISH in lymphoid follicles (arrows) in 85 days of age pig with PCV 2 SD.*

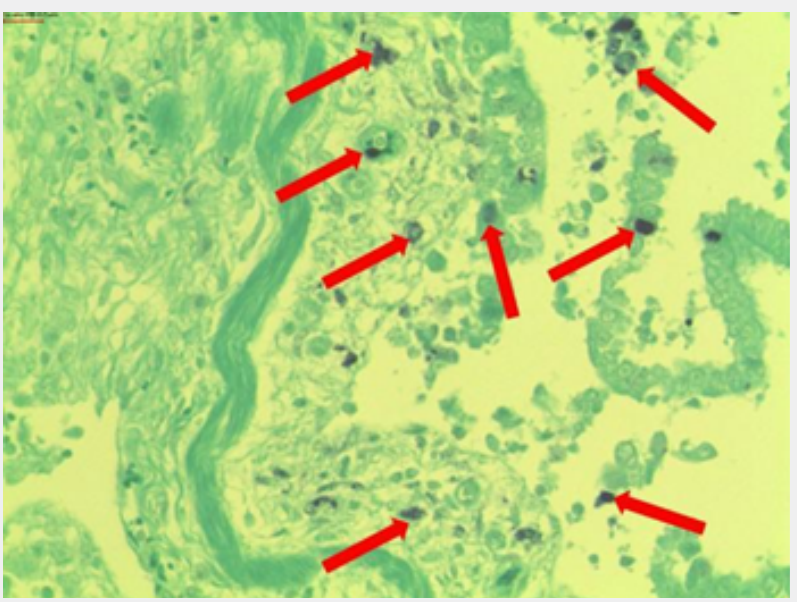

Figure 4: *Lung. PCV 2 nucleic acid confirmed by ISH in the cytoplasm of alveolar macrophages and bronchiolar epithelial cells (arrows) in 80 days of age pig with PCV 2 SD.*

Molecular Biological Studies

Conventional PCR result of 190 blood samples from pigs affected by PCV 2 SD show that DNA amplicons of 656 bp corresponding to those of the positive control were obtained (Figure 5). The percentage distribution of PCV 2 DNA positive 128 blood samples is presented in Figure 6. It is seen that blood samples from eleven age groups have been tested. The highest percent - 9.4 was found in pigs at 12 and 14 weeks of age. It will be noted that the adapted classical PCR with two primers, multiplying different regions of the genome of the virus to prove the infection in blood from infected animals, is a sensitive method of diagnosing PCV 2. In Figure 7 is shown a conventional PCR with specimens from lymph nodes of pigs. Amplification products (494bp) were found in five animals at 2, 8, 10 and 12 months of age. The same results were obtained in the corresponding blood samples. The results of the classical PCR and RT-PCR are consistent with the results of IHC and ISH, but the last two techniques identify the predilection sites for virus development. Therefore, it can definitely be said that the most sensitive methods for diagnosing PCV2 are IHC and ISH.

Figure 5: *DNA amplicons of 656 bp obtained by PCR from blood samples. M- marker = 100 bp; line: 4, 8 12, 16, 17, 21, 23, 24 - positive PCV 2 DNA; 1, 2, 3, 5, 6, 7, 9, 10, 11, 13, 14, 15, 18 - negative; line - 22 negative control from healthy pig; line 20 - positive control; line 25 - distilled water.*

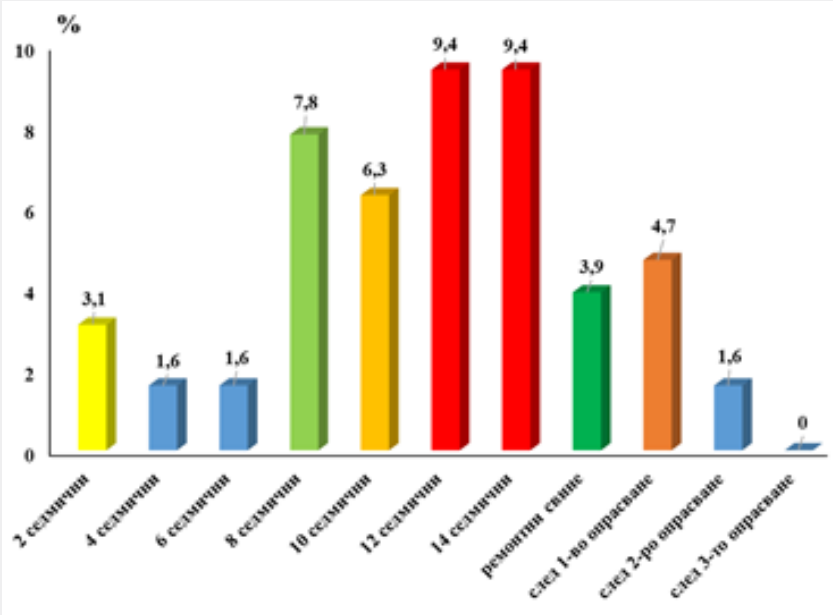

Figure 6: *Percentage distribution of positive for PCV 2 DNA 128 blood samples in pigs with PCV 2 SD in different age.*

Legend for eleven columns (left to right): *two-week pigs; four weeks; six weeks; eight weeks; 10 weeks; 12 weeks; 14 weeks; repair pigs; after first birth; after the second birth; after the third birth.*

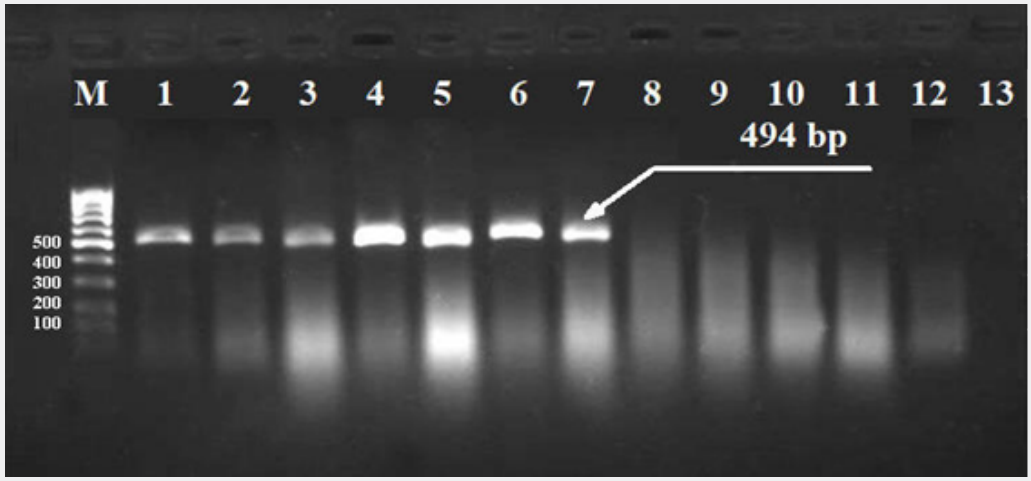

Figure 7: *DNA amplicons of 494 bp obtained by PCR from lymph nodes. M- marker = 100 bp; line: 1 and 2 positive controls; 3, 4, 5, 6, 7 - positive PCV 2 DNA; 8, 9, 10, 11, 12 - negative; line - 13 - negative control, distilled water.*

Upon sequencing the isolates PCV 2 is received data for differences in nucleotide and amino acid sequences of the capsid PCV 2 gene of Bulgarian isolates and reference strains (Tables 2, 3). We have found that the Bulgarian PCV 2 isolates "Han Asparuh 4" and "Han Asparuh 19" are identical in nucleotide sequences and form a separate branch of the reference isolates of PCV 2 genotype as they differ in the nucleotide sequences of the capsid PCV 2 gene in five positions and in the amino acid sequences in two positions. The phylogenetic analysis showed the exact positioning of these two isolates in a cluster 11 which was formed by the PCV 2b genotype of the PCV 2 isolates from the gene bank used in this assay.

Table 2: *Differences in nucleotide sequences of the PCV 2 capsid gene of Bulgarian isolates and Reference strains.*

Strain	Position in sequention				
	86	**105**	**402**	**466**	**471**
"2638 Lelystad", Netherlands	G	G	T	G	A
"1010", Scotland	C	G	A	C	G
"Ingezim Circo", Spain	A	G	T	G	A
"Ruse"	G	A	C	G	T
"Han Asparuh 4"	A	G	T	A	A
"Han Asparuh 19"	A	G	T	A	A

Table 3: *Differences in amino acid sequences of the PCV 2 capsid gene of Bulgarian isolates and Reference strains.*

Strain	Position in sequention				
	86	**105**	**402**	**466**	**471**
"2638 Lelystad", Netherlands	R	A	T	E	R
"1010", Scotland	T	S	I	D	R
"Ingezim Circo", Spain	K	A	T	E	K
"Rousse"	R	A	T	E	R
"Han Asparuh 4"	K	T	T	E	R
"Han Asparuh 19"	K	T	T	E	R

The third Bulgarian isolate "Rousse" differs in terms of nucleotide and amino acid sequences from the other Bulgarian isolates as well as from the reference isolates of PCV 2. This isolate differentiates a separate clone of genotype PCV 2b.

The phylogenetic tree, based on 32 sequences of the Bulgarian and reference isolates of PCV 2 is shown in Figure 8.

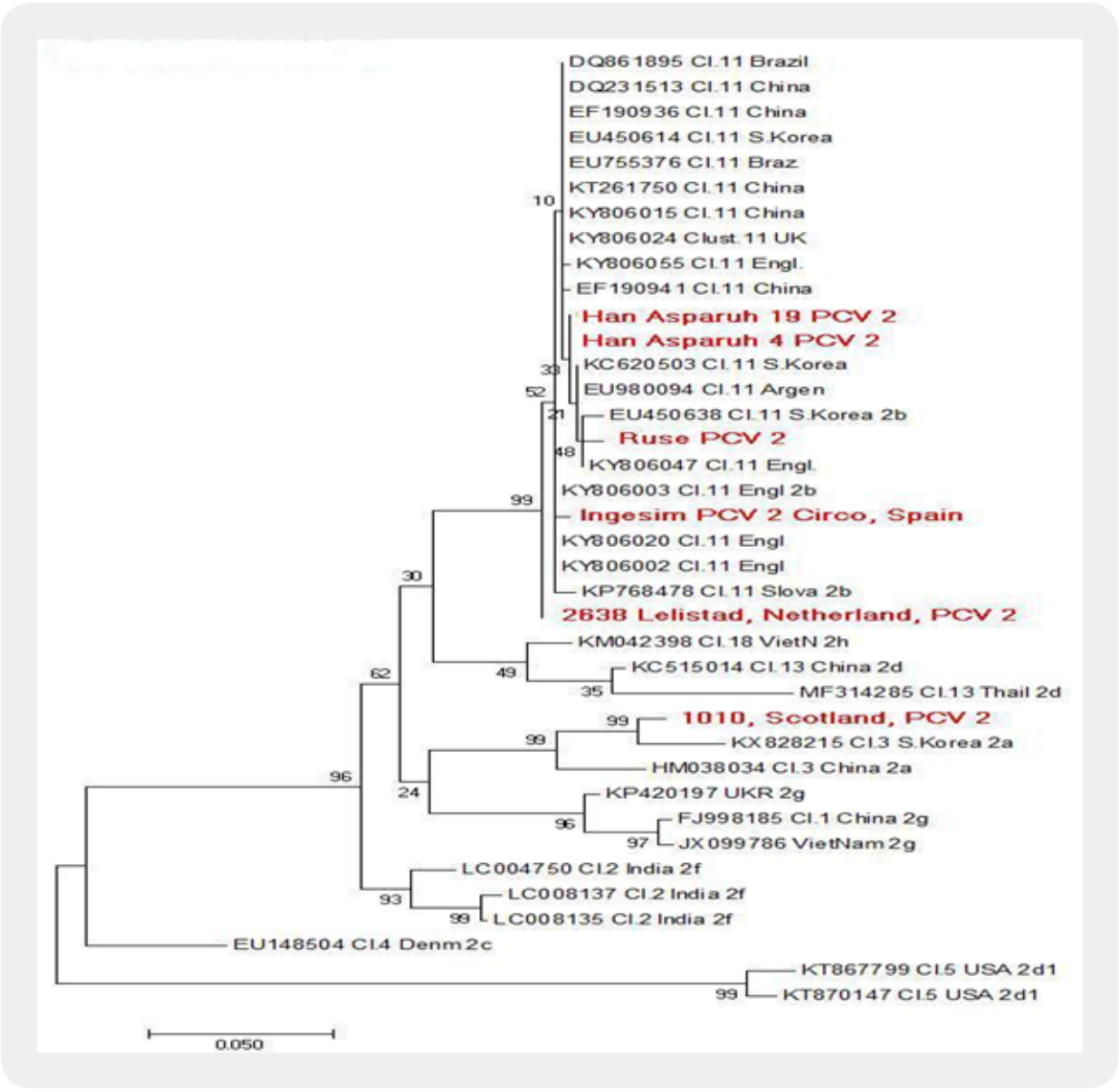

Figure 8: *Phylogenetic tree, based on 32 sequences of the Bulgarian and reference isolates of PCV 2.*

Histopathology

Most typical histological changes for circovirus infection were found in lymph nodes characterized by varying degrees of granulomatous inflammation (Figure 9A). Characteristically is also the formation of syncytial Langhans type giant cells (Figure 9B). Another feature is the partial or complete loss of normal lymph node structure and atrophy. It is due to partial or complete depletion of lymphocytes in the lymphoid follicles and in the para-cortical zone and their replacement by histiocytes. (Figure 10A). In some cases complete follicular and para-follicular destruction occurs (Figure 10B).

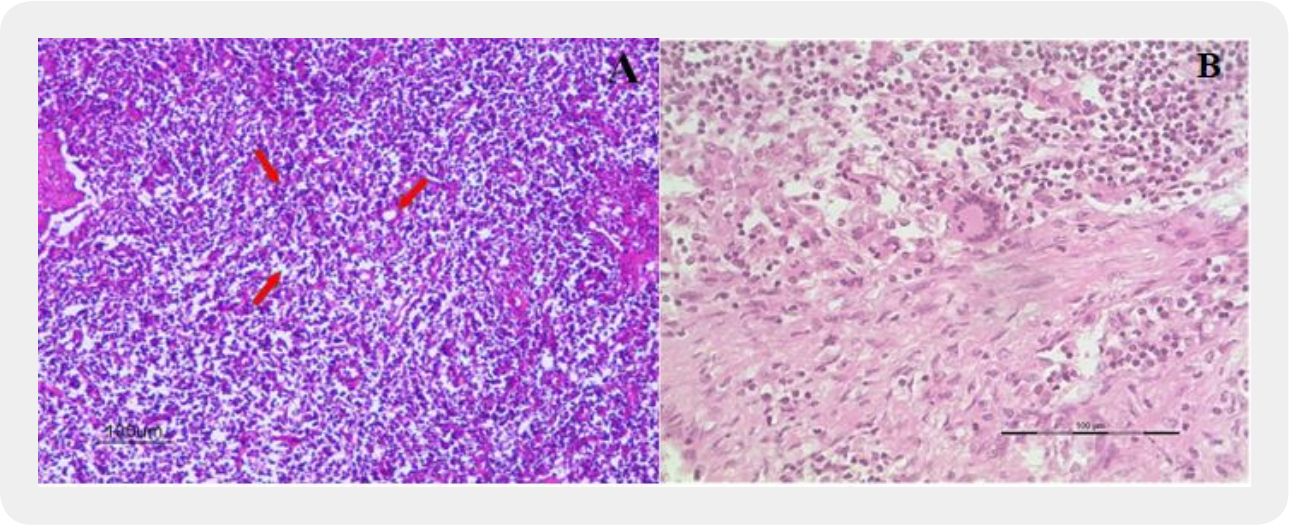

Figure 9: *Lymph node (A). Granulomatous inflammation and lymphoid depletion (arrows). Lymph node (B). Presence of multinucleated giant cells, Langhans type in 75 days of age pig. HE staining.*

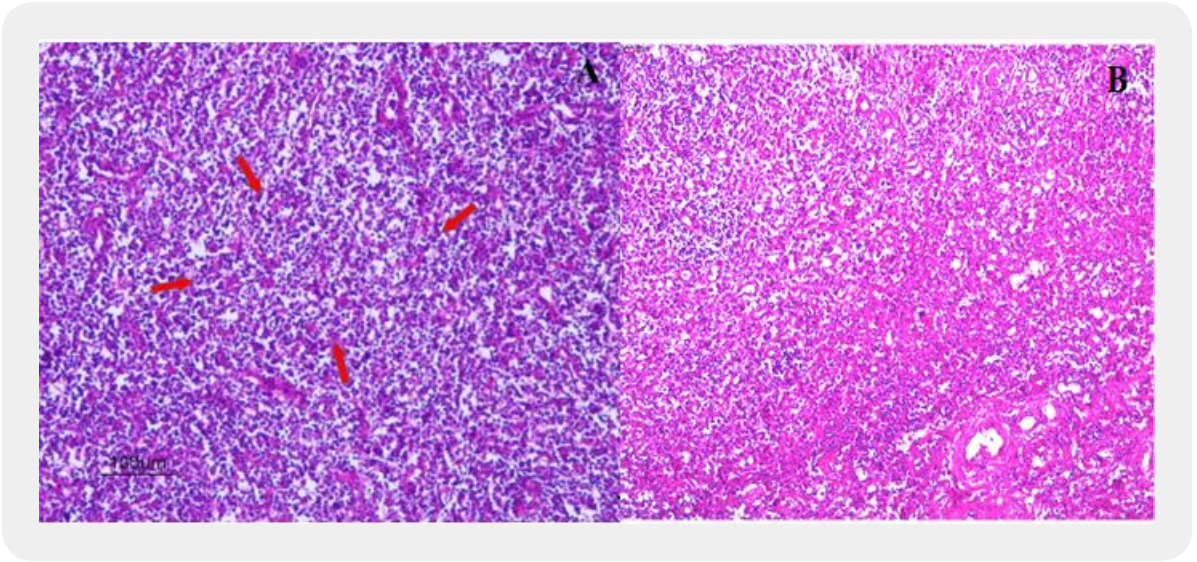

Figure 10: *Lymph node (A). Atrophy and lymphoid depletion in lymph follicles (arrows) in 81 days of age pig. Lymph node (B). Severe depletion in lymph follicles and parafollicular zone in 80 days of age pig. HE staining.*

In PCV 2 infected lungs, changes of varying severity were observed - purulent bronchopneumonia (Figure 11A), interstitial pneumonia (Figure 12), fibrinous pneumonia (Figure 13), and necrotizing pneumonia.

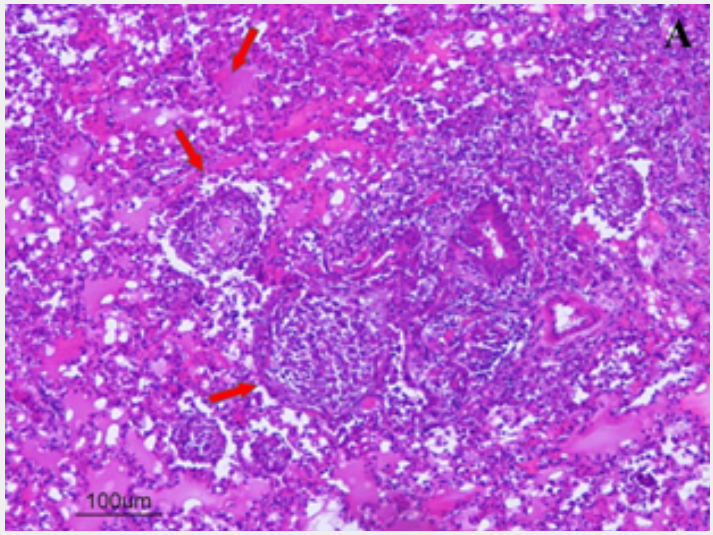

Figure 11: *Lung. Purulent bronchopneumonia in 87 days of age pig. Presence of purulent exudate in alveoles and bronchioles. HE staining.*

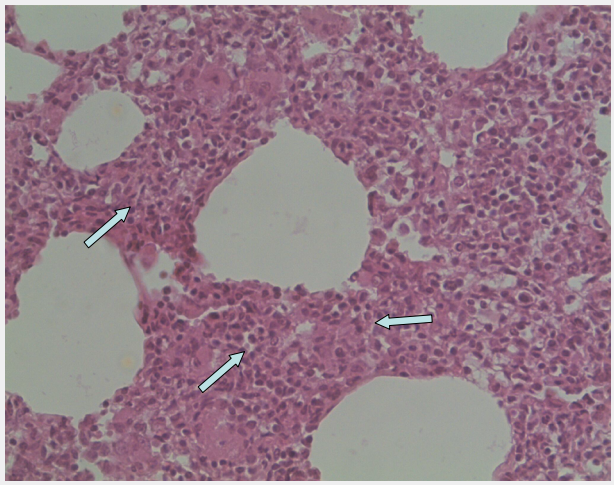

Figure 12: *Lung. Interstitial pneumonia, characterized by interstitial fibrosis and proliferation of mononuclear cells (arrows) in pig with PCV 2 SD. HE staining.*

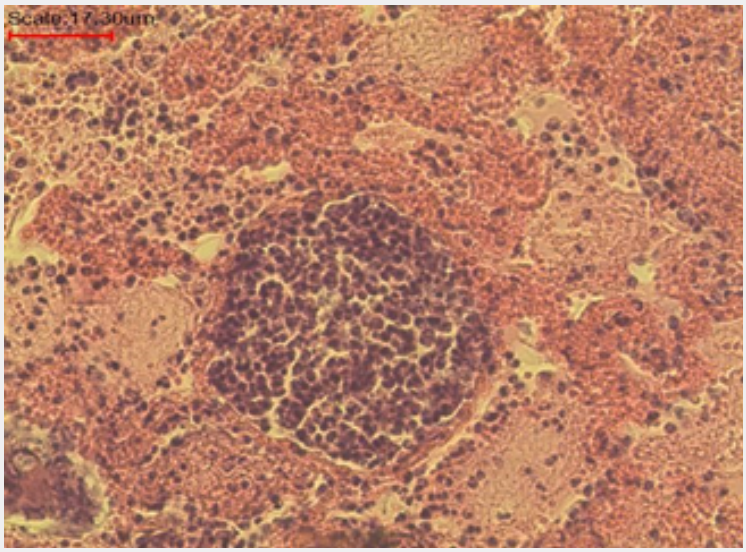

Figure 13: *Lung. Fibrinous pneumonia. Presence of plasma proteins and fibrin in bronchioles and alveoles, abundant exudate with neutrophils, lymphocytes, erythrocytes and edema in 90 days of age pig.*

Histological changes in the kidneys are shown in Figure 14, and in the skin in Figure 15.

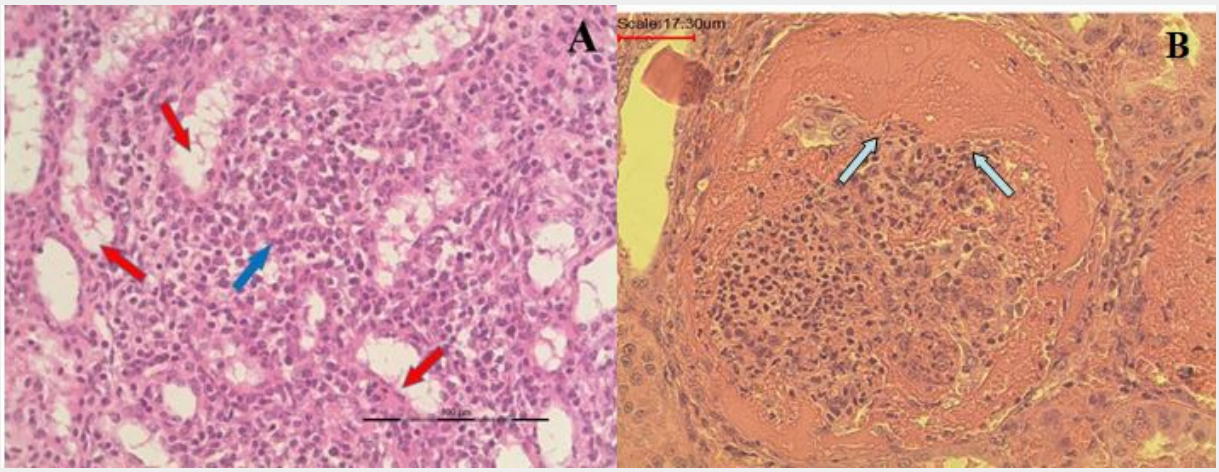

Figure 14: *Kidney (A). Degeneration of renal tubules - cell swelling and necrosis of epithelial cells (red arrows) and deposition of mononuclear cellular infiltrate in the interstitium (blue arrow). Kidney (B). Serous glomerulonephritis, accumulation of serous exudate beneath the capsule of renal glomerulus (arrows). HE staining.*

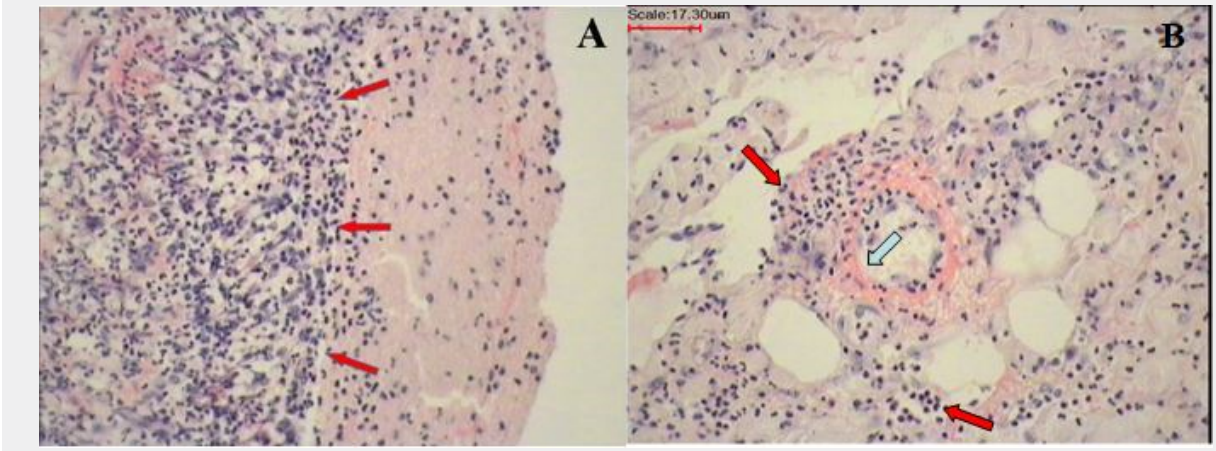

Figure 15: *Skin (A). Diffuse mononuclear lymphocytic infiltration in derma (arrows). Skin (B). Necrotizing vasculitis (blue arrow) and diffuse lymphocytic infiltration (red arrows). HE staining.*

Conclusions

♦ Circovirus infections, primarily PCV 2 are wide spread in the pig populations in Bulgaria.

♦ The predominant clinical syndromes in the affected pig herds are PCV 2 SI (subclinical infection), PCV 2 SD (systemic infection) and PCV 2 PDNS (porcine dermatitis and nephropathy syndrome).

♦ As the most susceptible to infection are the adolescent pigs aged 12-14 weeks.

♦ The most sensitive diagnostic methods for PCV 2 are immunohistochemisty (IHC) and in situ hybridization (ISH).

♦ The use of a polymer, with attached secondary antibodies, shorten the time of the immunohistochemical reaction, reduces the liklihood of non-specific reactions.

♦ Immunohistochemistry and in situ hybridization have shown that the most affected organs from PCV 2 infection are the lymph nodes, lymphoid organs, lungs and skin.

♦ The replication of the virus takes place in the lymphoid organs, which is confirmed by the presence of DNA established by ISH method.

♦ Adapted classical PCR with two types of primers, multiplying different parts of the viral genome, to prove infection in the blood of infected animals, is a sensitive method for diagnosing PCV 2.

♦ Sequencing of the Bulgarian isolates of PCV 2 revealed that they belong to the PCV 2b genotype.

♦ The studied Bulgarian isolates Han Asparuh 4 and Han Asparuh 19 are a separate branch from the reference isolates of PCV 2b, as they differ in the nucleotide sequences of the capsid PCV 2 gene in five positions, and in the amino acid sequences in two positions.

♦ The studied Bulgarian isolate Rousse differentiates separate branch of the PCV 2b genotype and differed in five positions in the nucleotide sequences of the other Bulgarian isolates Han Asparuh 19 and 4, and from the reference isolate 2638 Lelystad (Netherlands) in two positions. In the amino acid sequences Rousse differs from Han Asparuh 4 and Han Asparuh 19 and from Ingezim Circo (Spain) in two positions, and in five positions from the reference isolate 1010 (Scotland), belonging to the clone of genotype PCV 2a isolates.

Bibliography

1. Allan, G. M. & Ellis, J. A. (2000). Porcine circoviruses: a review. *J Vet Diagn Invest., 12*(1), 3-14.

2. Cunningham, G., *et al*. (2008). Proc. 20th IPVS Congr. Durban.

3. Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res., 32*(5), 1792-1797.

4. Ku, X., Chen, F., Li, P., Wang, Y., Yu, X., Fan, S., *et al*. (2017). Identification and genetic characterization of porcine circovirus type 3 in China. *Transbound Emerg Dis., 64*(3), 703-708.

5. Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol., 33*(7), 1870-1874.

6. Murtaugh, M. (2008). Molecular Epidemiology of Porcine Circovirus in the US Swine Herd. Research Report. Swine Health. Pork checkoff. NPB, 07-221.

7. Opriessnig, T., Meng, X. J. & Halbur, P. G. (2007). Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J. Vet. Diagn. Invest., 19*(6), 591-615.

8. Palinski, R., Pineyro, P., Shang, P., Yuan, F., Guo, R., Fang, Y., *et al.* (2017). A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure. *J. Virol., 91*(1), e01879-16.

9. Phan, T. G., Giannitti, F., Rossow, S., Marthaler, D., Knutson, T. P., Li, L., *et al.* (2016). Detection of a novel circovirus PCV3 in pigs with cardiac and multi-systemic inflammation. *Virol J., 13*(1), 184-192.

10. Rose, N., Larour Le Diguerher Blanchard, G. P., Le Dimma, M., Eveno, E., Jolly, J.P., Jestin, A. & Madec F. (2001). Serological profile of porcine circovirus type 2 (PCV2) in PMWS affected and nonaffected farms. Presented at the International Conference on ssDNA Viruses of Plants, Birds, Pigs, and Primates, St Malo, France.

11. Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodriguez-Arrioja, G. M., Kennedy, S., *et al.* (1999). Pathological, immunohistochemical, and in situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. *Journal of Comparative Pathology, 120*(1), 59-78.

12. Segalés, J. & Domingo, M., (2002). Postweaning multisystemic wasting syndrome (PMWS) in pigs. *A review. Vet Q., 24*(3), 109-124.

13. Segalés, J., Olvera, A., Grau, R. L., Charreyre, C., Nauwynck, H., Larsen, L., *et al.* (2008). PCV-2 genotype definition and nomenclature. *Veterinary Record, 162*(26), 867-868.

14. Shen, H., Liu, X., Zhang, P., Wang, L., Liu, Y., Zhang, L., *et al*. (2018). Genome characterization of a porcine circovirus type 3 in South China. *Transbound Emerg Dis., 65*(1), 264-266.

15. Sibila, M., Calsamiglia, M., Segales, J., Blanchard, P., Badiella, L., Le Dimna, M., *et al.* (2004). Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. *Am J Vet Res., 65*(1), 88-92.

16. Sofia, M., Billinis, C., Psychas, V., Birtsas, P., Sofianidis, G., Leontides, L., *et al.* (2008). Detection and genetic characterization of porcine circovirus 2 isolates from the first cases of postweaning multisystemic and wasting syndrome in wild boars in Greece. *J Wildl Dis., 44*(4), 864-870.

17. Stoev, S., Dinev, I., Manov, V., Grozeva, N. & Simeonov, R. (2016). Veterinary autopsy techniques and incineration. Iskra-M Publishing House, St. Zagora, Bulgaria.