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Pathomorphological and molecular investigations in naturally infected Chukar partridges (*Alectoris* Chukar) with *Histomonas meleagridis* and *Tetratrichomonas gallinarum*[1]

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ABSTRACT.- Ateş M.B., Harman H., Akçakavak G., Ozdemir O., Duran T. & Akbulut N.F. 2022. **Pathomorphological and molecular investigations in naturally infected Chukar partridges (*Alectoris* Chukar) with *Histomonas meleagridis* and *Tetratrichomonas gallinarum***. *Pesquisa Veterinária Brasileira 42:e07180, 2022*. Department of Pathology, Faculty of Veterinary Medicine, *Selçuk Üniversitesi*, Konya, Turkey. E-mail: mehmetburakates@selcuk.edu.tr

Histomonas meleagridis and *Tetratrichomonas gallinarum* are two protozoans responsible for mortality associated with typhlohepatitis in poultry. In this study, the etiology of high mortality in Chukar partridges suspected of infection with these agents was investigated pathologically and molecularly. Twelve healthy partridges during the laying period and 30 partridges that died due to disease and were included in the study. In blood analysis, increased levels of WBC, NEU, LYM, MONO, EO and BASO suggesting bacterial and/or parasitic infection; decreased levels of HGB, MCH and MCHC, which are markers of anemia; and increased AST and LDH levels, which are important for liver degenerations. In the liver, which is one of the most pathologically affected organs, multifocal necrosis foci that sometimes merge with each other and spread to large areas, and severe fibrino-necrotic typhlitis were detected. There was amyloid deposition in the space of Disse and vascular sinuses in the liver. PAS positive protozoal agents were observed in and around the lesioned areas. By PCR analyzes using specific primers, 11 of the samples were positive for *H. meleagridis* only, whereas 5 were positive for *T. gallinarum* only; 14 samples tested positive for both agents. Sequence analysis showed 100% identity between all samples resulting in positive PCR. In addition, *Escherichia coli* was produced in microbiological culture (27 of 30). When all the results were evaluated together, it was concluded that *H. meleagridis* and *T. gallinarum* and secondary *E. coli* may cause high mortality in partridges under lay stress.

INDEX TERMS: Hepatitis, histopathology, liver, partridges, *Alectoris* Chukar, typhlitis, *Histomonas meleagridis*, *Tetratrichomonas gallinarum*.

RESUMO.- [**Investigações patomorfológicas e moleculares em perdizes Chukar (*Alectoris* Chukar) naturalmente infectadas com *Histomonas meleagridis* e *Tetratrichomonas gallinarum*.**] *Histomonas meleagridis* e *Tetratrichomonas gallinarum* são dois protozoários responsáveis pela mortalidade associada à tiflohepatite em aves. Neste estudo, a etiologia da alta mortalidade em perdizes Chukar com suspeita de infecção por esses agentes foi investigada patologicamente e molecularmente. Foram incluídas no estudo 30 perdizes que morreram devido à doença e 12 perdizes saudáveis durante o período de postura. Na análise sanguínea, níveis aumentados de WBC, NEU, LYM, MONO, EO e BASO sugerindo infecção bacteriana e/ou parasitária; diminuição dos níveis de HGB, MCH e MCHC, que são marcadores de anemia; e aumento dos níveis de AST e LDH, que são importantes para as degenerações hepáticas. No fígado, que é um dos órgãos patologicamente mais acometidos, foram detectados focos de necrose multifocais que às vezes se fundem e se espalham

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para grandes áreas, e tiflite fibrino-necrótica grave no ceco. Houve deposição de amiloide no espaço de Disse e seios vasculares no fígado. Agentes protozoários PAS positivos foram observados dentro e ao redor das áreas lesionadas. Pelas análises de PCR com primers específicos, 11 das amostras foram positivas apenas para *H. meleagridis*, enquanto 5 foram positivas apenas para *T. gallinarum*; 14 amostras testaram positivo para ambos os agentes. A análise de sequência mostrou 100% de identidade entre todas as amostras resultando em PCR positivo. Além disso, *Escherichia coli* foi produzida em cultura microbiológica. Quando todos os resultados foram avaliados em conjunto, concluiu-se que *H. meleagridis* e *T. gallinarum* e *E. coli* secundária podem causar alta mortalidade em perdizes sob estresse de postura.

TERMOS DE INDEXAÇÃO: Hepatite, histopatologia, fígado, perdizes, *Alectoris* Chukar, tiflite, *Histomonas meleagridis*, *Tetratrichomonas gallinarum.*

INTRODUCTION

Histomonas meleagridis, an anaerobic protozoan with flagellate or amoeboid forms, causes histomoniasis, a severe disease in poultry. Consumption of embryonated eggs of the cecum nematode *Heterakis gallinarum* harboring *H. meleagridis* trophozoites or ingestion of worms that have eaten nematode eggs transmits the agent. Cecal necroses and hepatic confluent multifocal granulomas and/or necroses are characteristics of histomoniasis (black head disease). Partridges are also susceptible to histomoniasis, as are turkeys, which are known to be the most susceptible (Lund & Chute 1971).

Tetratrichomonas gallinarum has four anterior flagella and is a protozoan parasite found in the normal gastrointestinal microbiota of poultry. *T. gallinarum* can cause lesions in the liver and cecum, like histomoniasis. Although there is conflicting information regarding the pathogenicity of this organism in experimental infections, it has reported the circulation of a lethal strain of *T. gallinarum* in partridges (Amin et al. 2011, Liebhart et al. 2014).

The etiological agent of mortality cases shaped as a result of enzootic typhlohepatitis reported in birds has been identified as *H. meleagridis* (Tyzzer 1920). *T. gallinarum* infection, on the other hand, can cause typhlohepatitis in turkeys. The researchers found that lesions in the livers of infected birds can be discriminated macroscopically by protozoan type, and even the appearance of lesions caused by a mix infection of the two parasites is characteristic (Allen 1941, McDougald 2005). Mixed infections caused by *H. meleagridis* and *T. gallinarum* have been reported in blackhead disease, where the mortality rate reaches almost 100% (McDougald 2005). Apart from these two factors, there are other etiological agents that may cause similar clinical, macroscopic, and microscopic lesions (Supartika et al. 2006, Ateş et al. 2020). This situation leads to uncertainties in the diagnosis and can cause significant time and economic losses. For this reason, it is important to make differential diagnosis according to the etiological agents. In addition, the inability to fully reveal the complex etiology of such cases of typhlohepatitis, which causes great mortality with epidemics from time to time, or the delays experienced in this process makes it difficult to develop effective control and treatment strategies for the disease.

There is very limited and insufficient information on the mortality of partridges associated with typhlohepatitis. Therefore, in this study, *H. meleagridis* and/or *T. gallinarum* agents were investigated by advanced molecular techniques from samples taken from partridges. In addition, the exclusion of other factors that may cause typhlohepatitis or the identification of a mutual interaction, and similar lesions or pathomorphological differences according to the agents were investigated.

MATERIALS AND METHODS

Ethical approval. The compliance of the study with the ethical rules was approved by the Ethics Committee of the Experimental Animal Production and Research Center of the Faculty of Veterinary Medicine of Selçuk *Üniversitesi* (SÜVDAMEK) (Approval No: 2021/182).

Animals. 2550 chicks hatched with 85% hatching efficiency from 3000 eggs obtained from Chukar partridges raised in Konya Bahri Dağdaş International Agricultural Research Institute were raised in cages of 3m x 8m x 2m dimensions for 50 weeks. In April 1,400 breeding partridges were placed in cages measuring 1.2m x 1.15m x 6m with grid type litter at 90cm above the ground, and 200 were placed in a voiler measuring 40m x 20m x 5m at the end of the breeding period. There is 1km between the voiler and the breeding cages. Partridges were placed in both shelters according to the ratio of 3 females to 1 male and a 16-hour light – 8-hour dark period was applied. Water was provided ad libitum in both shelters via a nipple system, and feed was provided via automatic feeders. Deaths began in the voiler 45 days after the partridges were placed. In this study, 30 patient partridges, which are breeders and are in active spawning period, were used in these voilers in which deaths were observed. In addition, 12 partridges in the healthy cage, having the same characteristics and the same age, were determined as healthy controls.

Collection and analysis of blood samples. Blood samples were obtained from 12 partridges that exhibited evidence of sickness from the voiler, as well as 12 partridges that were reared in healthy breeding cages and showed no signs of disease or mortality. The blood of partridges that did not show any findings in necropsy in the patient group was not used. Blood collection was performed by cutting the vena jugularis with a sterile scalpel. Tubes with K3 EDTA were used for complete blood count, and tubes with clot activator were used for biochemistry analysis. Blood samples taken into tubes with clot activator were centrifuged at 3,000rpm for 10 minutes and serum was obtained. The obtained blood and serum samples were delivered to the laboratory within one-hour and analyzed. Blood biochemical analyzes were performed with Architect C8000 autoanalyzer (Abbott, USA), and complete blood count was performed with Cell DYN 3700 hemogram device (Abbott, USA).

Pathological examination. The deceased partridges were first subjected to systemic necropsies. After recording the macroscopic findings, samples were taken from the internal organs for smear sampling from the lesioned areas and for other examinations. Some of the samples were fixed in 10% formalin solution for 24 hours. The remaining parts were taken into cryotubes for molecular analysis and stored at -80°C. After fixation, all tissues were subjected to routine tissue processing and embedded in paraffin. Samples of 5µm thickness were taken from paraffin-embedded tissue blocks with a microtome (Leica RM2120). These samples were stained according to hematoxylin and eosin (HE), Periodic Acid-Schiff (PAS), Ziehl-Neelsen (ZN), Giemsa (GS), and Congo-red (CR) methods for microscopic examination (Luna 1968).

Bacteriology and parasitology. Swabs taken from the internal organs, especially the lesioned areas of the liver and cecum, were cultured on sheep blood agar and MacConkey agar (Remel, Lenexa/KS) for aerobic bacteria, and Brucella blood agar and phenylethyl alcohol agar for anaerobic bacteria. Bacterial species were determined from pure cultures on the basis of biochemical and phenotypic characteristics (Arda 2006). The presence of parasites in the smears prepared from the feces of the partridges was investigated microscopically.

PCR and sequence method. DNA was extracted from liver and caecum samples using the QIAamp DNA Mini kit (Qiagen, Valencia/CA) according to the manufacturer's instructions. PCR was performed using the Taq PCR master mix kit (Qiagen) with primer sequences for *Histomonas meleagridis* and *Tetratrichomonas gallinarum* (Table 1). Thermal cycling was performed with 35 cycles [30 seconds at 98°C; 30 seconds at 60°C (-0.5°C each cycle); 60 seconds at 72°C; 5 minutes at 72°C]. PCR was repeated three times and cases with positive signal at least twice were considered positive. Then the PCR products were purified and sequenced by the unidirectional Sanger sequencing method. The sequencing products were run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, California, USA). To identify the agent, the BLASTn suite search algorithm was used to compare the obtained sequences with reference homologous sequences obtained from the National Center for Biotechnology Information (NCBI) server[6].

Phylogenetic analysis. 18S rRNA sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) nucleotide database using the Basic Local Alignment Search Tool algorithm and protozoan strains were identified. Sequence data of 4 (4 of 7) randomly selected suspect isolates were aligned with previously published data on the 18S rRNA gene sequences of *H. meleagridis* and *T. gallinarum* isolates using the Clustal-W program (UCD Conway Institute, University College Dublin, Ireland). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGAX) software version 11.0 (Pennsylvania State University, University Park, Pennsylvania, USA) (Kumar et al. 2018). The maximum likelihood algorithm and Tamura-Nei model were used, and all gaps were excluded from the analysis. Also, branch support was determined using 1.000 bootstrap copies in the analysis.

Statistical analysis. Variation and homogeneity distributions of the data obtained from the measurement of hemogram, and blood biochemical parameters were controlled by Kolmogrov-Smirnov and Levene tests, respectively. The data determined to meet the parametric test conditions were analyzed with the one-way ANOVA post-hoc Duncan test (SPSS Inc. for Windows® version 25.0, Chicago/IL, USA). The results were presented as mean ± standard error of mean (SEM). A value of $P<0.05$ was considered significant.

Table 1. Nucleotide sequences used in the present study

Primer	Sequences	Amplicion	Accession number
Hmf	5'-GAAAGCATCTATCAAGTGGAA-3'	574	AF293056
Hmr	5'-GATCTTTTCAAATTAGCTTTAAA-3'		
Tgf	5'-GCAATTGTTTCTCCAGAAGTG-3'	526	AF124608
Tgr	5'-GATGGCTCTCTTTGAGCTTG-3'		

Hm = *Histomonas meleagridis,* Tg = *Tetratrichomonas gallinarum.*

[6] Available at <http://www.ncbi.nlm.nih.gov/BLAST/> Accessed on Apr. 7, 2022

RESULTS

Blood analysis results

The results of hemogram and biochemical analysis from blood taken from partridges are presented in Table 2 and 3. When hemogram results are evaluated, white blood cell (WBC), neutrophil (NEU), lymphocyte (LYM), monocytes (MONO), eosinophil (EOS), hemoglobin (HGB), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) levels a significant increase was determined compared to the healthy control ($P<0.05$). Although there were changes in basophil (BASO), erythrocyte (RBC), hematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width (RDW), platelet (PLT) levels compared to healthy control, this situation was not statistically significant ($P>0.05$) (Table 2). In terms of biochemical results, a significant increase was found in aspartate transaminase (AST) and lactate dehydrogenase (LDH)($P<0.05$), while the difference was insignificant in alanine transaminase (ALT) and alkaline phosphatase (ALP) ($P>0.05$, Table 3).

Pathological results

The first notable finding in the necropsies of partridges was prominent eye sockets, dehydration, cachexia, and tangles in

Table 2. Hemogram results*

	Diseased	Healthy	Unit	*P* value
WBC	49.87 ± 7.82	18.76 ± 1.44	10e3/µ	0.02*
NEU	9.62 ± 3.41	1.34 ± 0.21	10e3/µ	0.02*
LYM	39.20 ± 10.28	15.29 ± 2.03	10e3/µ	0.02*
MONO	1.23 ± 0.32	0.18 ± 0.04	10e3/µ	0.001*
EOS	3.91 ± 1.26	0.51 ± 0.10	10e3/µ	0.023*
BASO	1.67 ± 0.91	1.44 ± 0.76	10e3/µ	0.79
RBC	2.31 ± 0.18	2.86 ± 0.30	10e6/µ	0.228
HGB	12.55 ± 1.17	17.16 ± 1.55	g/dl	0.039*
HCT	34.98 ± 2.66	45.05 ± 4.52	%	0.065
MCV	152.62 ± 5.35	158.50 ± 2.12	µm3	0.47
MCH	53.96 ± 1.50	60.61 ± 1.45	pg	0.008*
MCHC	35.51 ± 0.92	38.26 ± 0.59	g/dL	0.03*
RDW	15.40 ± 1.10	13.18 ± 0.97	%	0.155
PLT	5.80 ± 2.21	2.96 ± 0.60	10e3/µl	0.302

* The statistical difference according to the One way Anova post-hoc Duncan test is significant ($P<0.05$); WBC = white blood cell, NEU = neutrophil, LYM = lymphocyte, MONO = monocytes, EOS = eosinophil, BASO = basophil, RBC = erythrocyte, HGB = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RDW = red blood cell distribution width, PLT = platelet.

Table 3. Biochemical results*

	Diseased	Healthy	*P* value
AST (U/L)	733.25 ± 74.17	520.33 ± 48.87	0.03*
ALT (U/L)	23.09 ± 3.30	19.42 ± 2.40	0.72
ALP (U/L)	243.18 ± 65.12	334.28 ± 95.40	0.24
LDH (U/L)	1,560.83 ± 224.23	750.20 ± 38.32	0.04*

AST = aspartate transaminase, ALT = alanine transaminase, ALP = alkaline phosphatase, LDH = lactate dehydrogenase; * The statistical difference according to the One way Anova post-hoc Duncan test is significant ($P<0.05$).

the feathers. There were no abnormal findings in the mouth, tongue, or esophagus. Macroscopically, the most important findings were concentrated in the liver and intestinal segments. Collapsed necrotic foci, 1-10mm in size, surrounded by a yellow-white ring and with pink-reddish centers were observed in the livers of all partridges necropsied. It was determined that these foci sometimes merged with each other and spread over large areas. In some cases, gray-white, slightly raised nodular foci were also detected. Exudate with a yellowish solid consistency accompanied by hyperemia in the small intestine segments was noted. The caecum of all partridges was found to be thickened due to fibrin accumulation. A debris with a caseous core obstructed the cecum, almost like a plug, and was accompanied by hemorrhage. It was determined that this exudate was firmly adhered to the cecal mucosa. In addition, similar degenerative findings were observed in the cecal tonsils (Fig.1-4). Adhesions (synechia) were evident between the organs in the abdominal cavity and

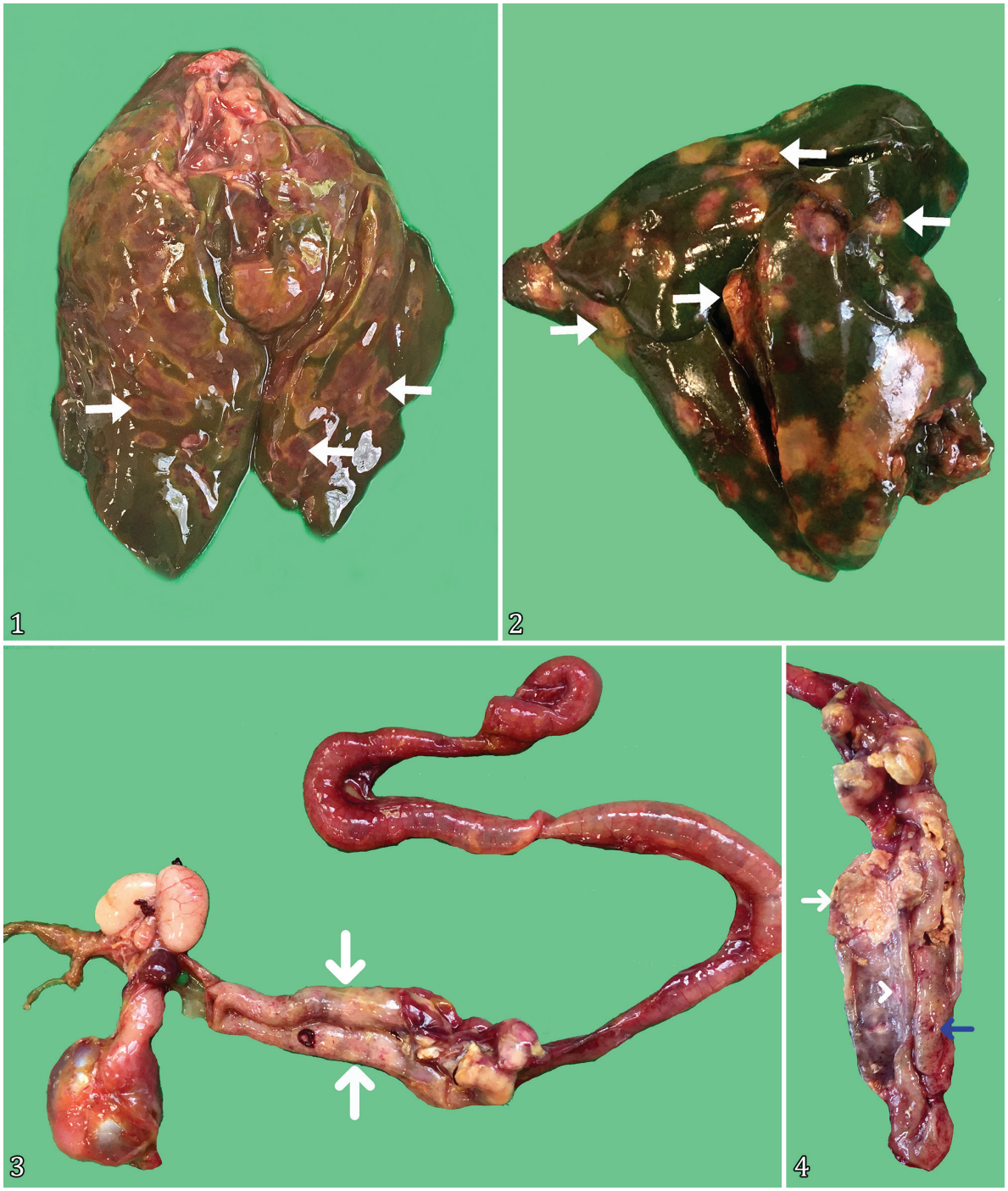


Fig.1-4. Macrophotographs. (**1**) Slightly collapsed necrosis foci surrounded by a yellow-white ring in the liver (arrows). (**2**) Gray-white, slightly raised nodular necrotic foci in the liver (arrows). (**3**) Increase in thickness due to fibrin accumulation in the caecum (arrows). (**4**) Fibrin debris (white arrow), ulcer (arrowhead), haemorrhages in the caecum (blue arrow).

the peritoneum. Slight enlargement of the spleen and bursa Fabricius, and congestion in the lungs of some partridges were observed. No abnormal finding was found in the macroscopic examination of the brain and nerves.

The histological examination revealed multifocal localized coagulation necrosis in the liver, sometimes accompanied by fibrin with necrotic nuclei and occasionally merging and extending to wide areas. Numerous spherical or oval-rounded centers with slightly eosinophilic 8-20-micron size protozoal trophozoites mixed with these necrotic areas were identified. Some of these structures were found to be PAS positive. Lymphocyte, epithelioid histiocyte, multinucleated giant cells and heterophile granulocyte infiltration were observed around the necrosis and protozoal deposits. In addition to periportal inflammatory cell accumulations in the liver, areas of necrosis spreading from here to the parenchyma were determined. In addition, accumulation of hyalinized-eosinophilic material was detected in the space of Disse and vascular sinuses (16 of 30). This material, which was observed as brick red in Congo-red staining, was found to be amyloid (Fig.5-7).

Intense infiltration of mononuclear cells and heterophile granulocytes was observed in the lamina propria of the intestinal sections. There were ulcer areas reaching up to the serosal layer in the caecum. In all layers of the caecum, thickening was evident with foci spreading over large areas in transmural fibrino-necrotic character. An amorphous eosinophilic material consisting of abundant shed epithelial cells, inflammatory cells and necrotic material was encountered in the lumen. Fibrinous exudate adhered to the serosa layer was evident (Fig.8). Numerous protozoal agents were identified in these necrotic areas as described in the liver (Fig.9). Also, various developmental forms of *Histomonas meleagridis* were found in smears made from lesioned areas (Fig.10).

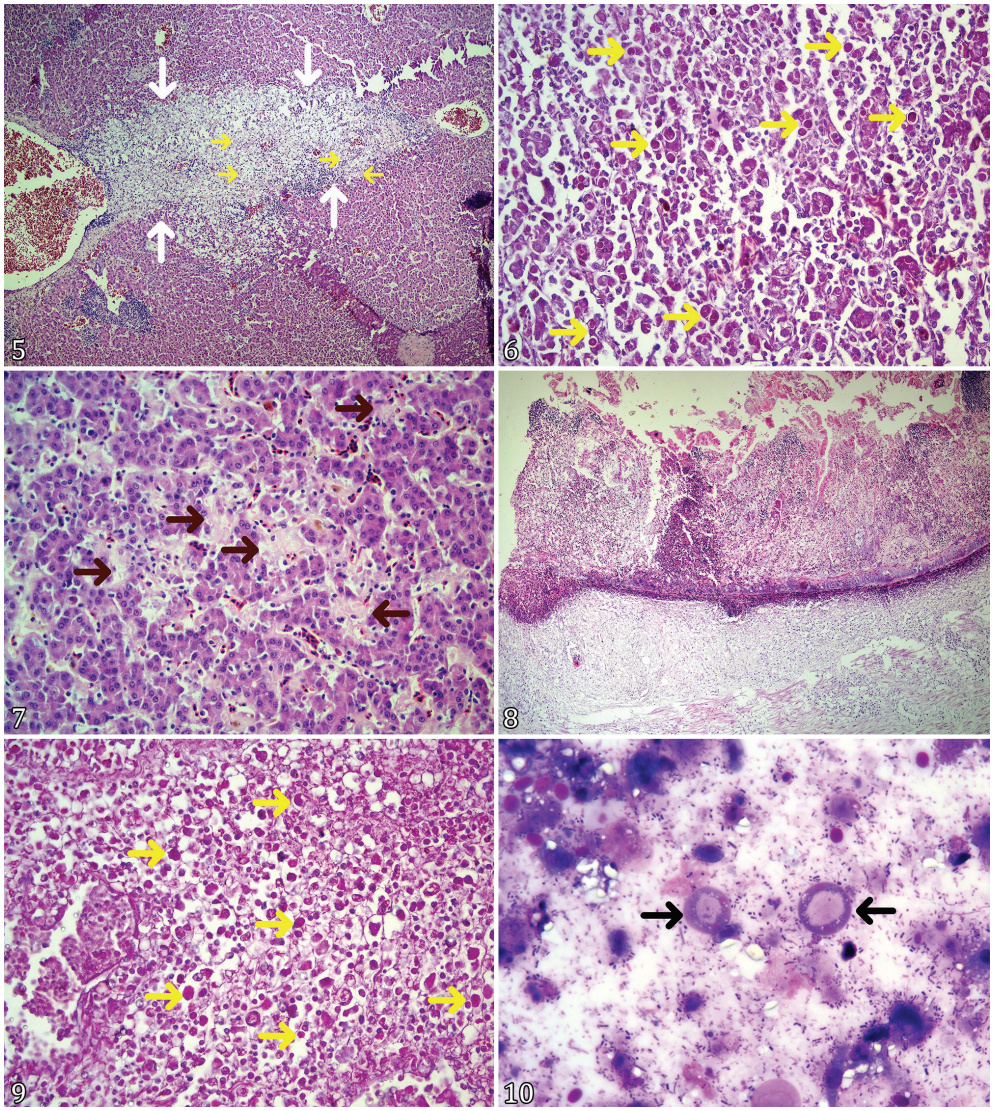


Fig.5-10. Microphotographs. (**5**) Liver. Necrosis begins in the portal area and spreads to the parenchyma (white arrows) and protozoal trophozoites (yellow arrows). HE, obj.10x. (**6**) Liver. PAS positive protozoal trophozoites (yellow arrows). PAS stain, obj.20x. (**7**) Liver. Amyloid deposition in the space of Disse. HE, obj.10x. (**8**) Caecum. Fibrino-necrotic typhlitis. HE, obj.10x. (**9**) Caecum. PAS positive protozoal trophozoites (yellow arrows). PAS stain, obj.20x. (**10**) *Histomonas meleagridis* (black arrows) in smear samples prepared from the caecum. Giemsa stain, obj.40x.

Lymphoid depletion and small foci of necrosis were detected in the spleen (4 of 30) and bursa Fabricius (7 of 30). Hyperemia in the interalveolar septum in the lungs and oedema in the alveoli (6 of 30) and lymphoid hyperplasia were observed (1 of 30). Hydropic degeneration (8 of 30) was observed in tubular epithelial cells in kidneys, and protein-rich fluid (4 of 30) was observed in tubulus lumens. In the brain, hyperemia (4 of 30) was detected in the meninges. No acid-resistance bacteria were found in the Ziehl Neelsen-stained sections.

Bacteriological and parasitological results

In bacteriological cultures, *Salmonella* spp. and *Yersinia* spp. were not found, but *Escherichia coli* (27 of 30) was produced from liver and caecum samples. In the examination of the smear samples prepared from the feces, structures showing the morphology of protozoan parasites were not found.

PCR and sequence results

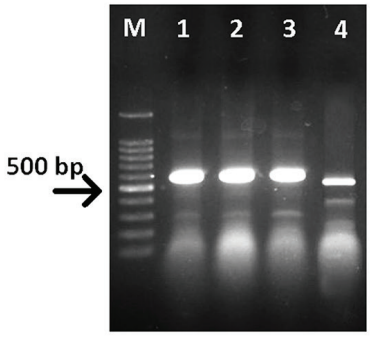
Primers specific for the 18S rRNAs of *H. meleagridis* and *T. gallinarum* were used for the analyses. All samples from frozen liver and caeca were positive for at least one agent. Eleven of the positive samples were only *H. meleagridis*; 5 were only *T. gallinarum*; both agents were detected in 14 (Fig.11-12). Sequence analysis showed 100% identity between all samples resulting in positive PCR. Sequence identity of ≤98% was determined with different isolates of *H. meleagridis* and *T. gallinarum* isolated from different bird species. The phylogenetic tree produced as a result of the analyzes is presented in Figure 13.

DISCUSSION

In this study, it was aimed to investigate the agents that may cause typhlohepatitis in mortality of partridges, to help clarify their pathogenesis and to examine *Histomonas meleagridis* and/or *Tetratrichomonas gallinarum* by molecular tests. In the necropsy of affected partridges, the intensification of the lesions especially in the caecum and liver, the encountering of many protozoal parasites around or inside the lesioned areas in histopathological and smear examinations have motivated the researchers to investigate these parasitic agents.

The character of necrosis observed especially in the liver in fatal typhlohepatitis cases is important to determine the etiology of the disease. As a matter of fact, when the lesions observed in the livers are evaluated, similar lesions in poultry are concentrated in *M. tuberculosis* (Gonzalez et al. 2002), *Escherichia coli* (Ateş et al. 2020), *H. meleagridis* (Clarke et al. 2017), *T. gallinarum* (Liebhart et al. 2014) and some fungal agents (Supartika et al. 2006). Researchers reported that the macroscopic lesions observed in such cases contributed to the etiological diagnosis (Allen 1941, Supartika et al. 2006). It has been reported that avian tuberculosis has caseified nodules that tend to spread to the spleen and bone marrow (Landman & van Eck 2017, Yavuz et al. 2021). In the present study, nodules with caseified foci were not observed in the bone marrow, spleen, and liver, and it was partially differentiated from tuberculosis in macroscopic examination. On the other hand, in a study in which only *E. coli* was detected, it was emphasized that lesions in the cecum were milder, as well as nodular structures that spread to the parenchyma in the liver and slightly protruded from the surface (Ateş et al. 2020). *Tetratrichomonas* are characterized by cheesy remnants of bloody tissue in the caecum and granular, cream-colored, well-demarcated necrotic areas in the liver that are flush with or slightly protruding from the surface. Although the lesions are very similar when compared with histomoniasis, it has been stated that the lesions in the liver in tetratrichomoniasis are distinguished by their granular structure and not having a slight collapse from the surface (Allen 1941). In the current study, it was found that the macroscopic lesions in some cases were similar to those attributed to histomoniasis, while others were similar to lesions caused by *Tetratrichomonas*. In terms of macroscopic examination findings, partial dissociation from the diagnosis of bacterial typhlohepatitis was supported by the observation of protozoa in the microscopic examination of smears made from the lesioned areas.

Microscopic examination revealed diffuse necrosis in the liver and lower digestive tract, especially in the caecum. Many PAS-positive protozoon agents were determined in the intra and perilesional areas. In addition, fungal hyphae were not observed in PAS and HE staining. The absence of any acid-resistance bacteria in ZN staining also completely excluded the diagnosis of tuberculosis. The milder distribution of the lesions in other internal organs was accepted as an indication that a hematogenous spread was likely. It was emphasized that the lesions formed by histomonads and



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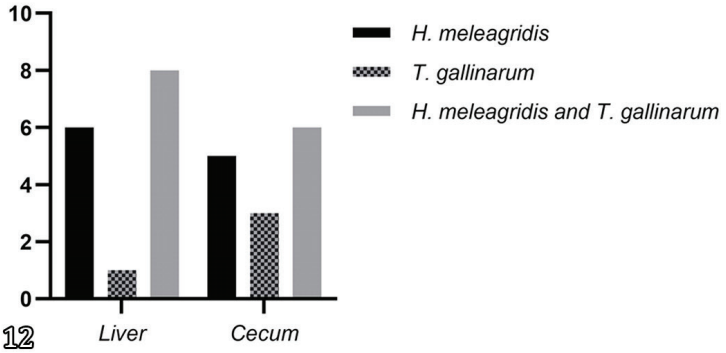

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Fig.11-12. PCR results. (**11**) Hmf/Hmr (574 bp) and Tgf/Tgr (527 bp) in each PCR reaction. The PCR products were electrophoresed on a 2% agarose gel and seen under UV light after being stained with ethidium bromide. M = molecular size marker (100 bp ladder); 1, 2 and 3rd bands = *Histomonas meleagridis* and *Tetratrichomonas gallinarum* positive; 4th band = *T. gallinarum* positive. (**12**) Graphical display of positive reaction numbers.

tetrarichomonads are very similar microscopically and it is very difficult to distinguish between the two agents (Liebhart et al. 2014). It has also been stated that a definition based on the morphology of parasites observed by histopathology is unreliable (Liebhart et al. 2014). Thus, the morphological similarity of these parasites in the tissues necessitates additional examinations to make the diagnosis.

A substantial amount of homogenous eosinophilic material accumulation in the Disse space of the liver was found as amyloid by Congo-red and HE staining. In poultry, amyloid is a pathological condition characterized by abnormal protein deposition in tissues, inducible by both infectious and non-infectious causes or by chronic inflammation (Crespo 2020). Cases of generalized or localized amyloidosis have been reported previously in partridges (McMartin et al. 1996, Kapakin et al. 2010). It is known that the only type of amyloid observed in poultry is amyloid A, which is encountered in recurrent or chronic infections (McMartin et al. 1996, Kapakin et al. 2010, Özdemir et al. 2013, Hauck et al. 2019). In a study investigating the mortality of partridges with typhlohepatitis and amyloidosis, it was reported that a high rate of *T. gallinarum* sequence homology was obtained (Hauck et al. 2019). Molecular positive detection of *T. gallinarum* in most of the cases in which amyloidosis was observed, both based on this study and in the current study, suggests that the underlying cause of amyloid observed in typhlohepatitis cases may be due to the pathogenicity of *Tetratricomonads* present in the normal gastrointestinal microbiota of poultry.

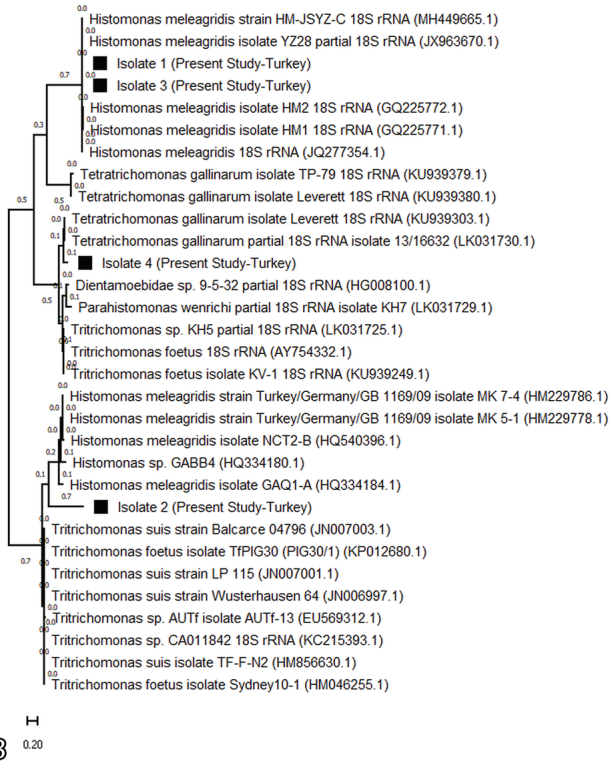

Fig.13. The maximum likelihood tree of parabasalids based on the sequences of the 18S rRNA genes. The maximum likelihood algorithm and Tamura-Nei model were used. Newly determined sequences are given in bold box. In parentheses, the GenBank accession numbers are listed. Scale bar indicates 0.20 substitutions (corrected) per base pair.

The lesions in the present study were observed to be characterized by necrosis and surrounding dense lymphocytes, epithelioid histiocytes, multinucleated giant cells and heterophile granulocyte infiltration. It has been reported that this type of granulomatous lesions can also be caused by *E. coli* in poultry (Ateş et al. 2020). However, it has been reported that coligranulomas are characterized by caseous or fibrino-heterophilic foci and there is no protozoan factor in or around the lesions (Supartika et al. 2006, Ateş et al. 2020). In addition, healthy birds are highly resistant to virulent strains of *E. coli* that can cause infection under certain stress conditions (Nolan et al. 2020). On the other hand, it has been reported that *Histomonads* reach full virulence in the presence of some bacteria such as *E. coli* in the caeca and are avirulent in birds without bacteria (Franker & Doll 1964, Springer et al. 1970). For this reason, *E. coli*, may have contributed to the increase in the severity of protozoa-borne infection in the present case.

There is no study in the literature in which the reference ranges of hemogram parameters in Chukar partridges are determined. Therefore, comparisons were made according to healthy partridges. The levels of WBC, neutrophil, lymphocyte, and monocytes that increased in infected partridges were higher in sick partridges compared to healthy partridges. It is known that these parameters increase in cases of infection (Noyan 2011). It has been reported that blood eosinophil levels increase in parasitic infections and allergies (Noyan 2011). In the study, eosinophil levels were eight times higher in sick animals than in healthy animals. Decreases in HGB, MCH and MCHC levels in the patient group suggested that the current infection caused anemia.

Since reference ranges for Chukar partridges could not be found in the literature in biochemical parameters, comparisons were made between sick and healthy animals. While the AST enzyme, which shows an increase in liver damage, was higher in the patient group, there was no statistical difference in the ALT enzyme, which also gives an idea about liver damage (Lehninger et al. 2005). Serum LDH levels are accepted as an indicator of cell damage and necrosis (Van Wilpe et al. 2020). Due to the liver damage observed in the study, serum LDH levels were determined to be 2 times higher than in healthy partridges. ALP is particularly abundant in the liver, bone and placenta. It has also been reported that it plays an important role in the hepatobiliary and skeletal systems, and serum ALP levels have been used as a useful parameter in liver and bone problems for a long time (Kim et al. 2020). In the study, no difference was found between the groups in terms of ALP. Although liver damage was detected at necropsy, no macroscopic lesions were observed in the bones. Therefore, according to the serum ALP levels determined in the study, it was interpreted that the existing mix infection did not cause a significant damage to the bone system in Chukar partridges. There was no significant change in hemogram results or biochemical parameters when *H. meleagridis*, *T. gallinarum*, or both were observed separately.

The specific primer sets utilized in this investigation did not exhibit any cross-reactions for *H. meleagridis*, while *Trichomonas gallinae* was found in only one PCR product in *T. gallinarum*, with no non-specific binding in other samples (Grabensteiner & Hess 2006). The diagnostic tests used to

distinguish *H. meleagridis* and *T. gallinarum* so far are very limited and mostly rely on microscopic examination of feces or in vitro culture methods. Although pathological studies have a key role in mortality associated with typhlohepatitis, especially after some additional staining, they are insufficient to differentiate between these two agents. For this reason, confirmation by molecular characterization is a very important step in order to establish the diagnosis quickly in this type of epidemics, which are rarely observed in partridges. In the current study, specific primer sets used in the separation of these two factors with high sensitivity for other birds were used in partridges, and the accuracy of positive PCR results was demonstrated by the analyzes of the loci sequenced by sequencing and phylogenetic analysis.

T. gallinarum, which is found in the normal intestinal microbiota, is still debated as to whether it is harmful in other poultry species, and outbreaks in partridges are infrequent (Amin et al. 2011, Liebhart et al. 2014). Cepicka et al. (2005) found the high molecular polymorphism of *T. gallinarum* and suggested that different subspecies may differ in their ability to cause pathological manifestations. Similarly, in the current study, fatal typhlohepatitis was observed in cases where *T. gallinarum* was detected alone, which was interpreted as the pathogenic effects of this agent. Perhaps, as in the cases in the current study, the immunosuppression occurring in co-infections with *E. coli* and the appropriate intestinal microenvironment may have contributed to the pathogenic character of *T. gallinarum*.

CONCLUSION

In the light of clinical, pathological, haematological, biochemical, and molecular data, it was determined that the high mortality, cachexia, and typhlohepatitis observed in partridges in the present study were caused by pathogenic strains of *Histomonas* and *Tetratrichomonas*. It was thought that the spawning stress of partridges in the egg laying period and coliform bacteria simultaneously contributed to the severe course of the disease. Complex etiology must be taken into consideration in order to prevent losses in typhlohepatitis of partridges, which has a high mortality rate.

Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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