Pathoanatomical and blood biochemical investigations in chicks, challenged with *Escherichia coli* on the background of a pre-existing *Eimeria* infection

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**SUMMARY**

Because of high frequencies of colibacillosis and coccidiosis in poultry and their eventual coexistence, this study was conducted for investigating plasma enzyme activities (ASAT, ALAT and ALP) in association with the severity of pathoanatomical findings in chickens with mono-infections (with *E. coli* or *E. tenella* only) or co-infection. For that, 160 broiler chickens were randomly divided into 4 equal groups according to the pathogen administration: birds from the group I (mono-infected) and from the group III (co-infected) were orally infected with 8.10⁴ sporulated *E. tenella* oocysts when they were 12 days old and *E. coli* (serotype O78, 10⁷ cfu) was intraperitoneally injected to chickens from the group II (mono-infected) and from the group III when they were 16 days old, whereas the group IV served as negative control group. Blood samples were collected and animals were slaughtered when they were 20 days old. Eimeria infected chickens exhibited haemorrhagic inflammatory lesions associated with dystrophic and necrotic processes principally in the caecum but also in liver and kidneys, in heart and in brain and cerebellum while in birds receiving *E. coli*, a fibrinous air-sacculitis, perihepatitis or pericarditis, and a gallbladder inflammation were also noticed and that in the case of mono-infection, caecum and brain lesions were absent. By contrast, all pathological findings were present and exacerbated in co-infected chickens. The plasma ASAT and ALAT activities were significantly increased in all infected chickens whereas ALP activities were absent. By contrast, all pathological findings were present and exacerbated in birds co-infected. These results suggest that pre-invasion with *E. tenella* aggravates the colibacillosis and that this situation would be under-estimated in clinical practice.

**Keywords**: Broiler chickens, *E. tenella*, *E. coli*, co-infection, inflammation, fibrin thrombus, ASAT, ALAT, ALP.

**Introduction**

Colibacillosis in poultry occurs as acute, subacute or systemic disease and affects broilers at the age of 2-4 weeks [32], the commonest age of eimeriosis occurrence in chickens [31]. Two primary clinical syndromes of disease manifestation are known: acute septicaemia accompanied with high mortality in small chickens and subacute fibrinopurulent serositis and/or septicaemia in 2 to 8-week-old birds [6].

*Escherichia coli* belongs to the normal intestinal microflora in mammals and birds. The pathogen *E. coli* isolates (EPEC) for birds belong to O sero-groups, and Sojka and Carnaghan...
[41] reported for the first time that three of them, namely O1, O2 and O78, were the commonest isolates (15 to 61% of all isolates) in case of avian colibacillosis in different countries worldwide [4, 7, 9, 13, 19]. However, most investigators believe that probably, EPEC were not related to intestinal diseases such as avian enteritis, although enterotoxigenic 

*Escherichia coli* have been associated with occurrence of diarrhoea in chickens [1, 23, 43, 46, 49]. The colibacillosis in birds is diagnosed on the basis of clinical signs and the presence of airsacculitis, pericarditis and peri-hepatitis. Further, the diagnosis is confirmed by isolation of the Escherichia coli pathogen agents.

The protozoan parasites of the *Eimeria* genus reproduced in the avian gastro-intestinal tract, causing tissue damage that result not only in reduction of weight gain, but also in increased susceptibility to various microbial pathogens [34]. In bacteria-free chickens, infected with surface-sterilized *E. tenella* oocysts, clinical signs of coccidiosis have not been observed unless two or more intestinal bacterial were present [21, 38, 47-49]. Apparently, intestinal bacteria were necessary for the development of the typical caecal coccidiosis in chicks. As early as in 1909 and 1910, HADLEY [16] and FANTHAM [11] established bacterial infection of viscera in chickens, when bacterial challenge preceded the parasite infection. Frequently, eimeriosis in birds is observed, when bacterial challenge preceded the age without antibiotics and coccidiostatics. The rearing conditions minimized the possibility for infection with either *Eimeria* or pathogen *E. coli* (EPEC).

The chickens were divided into 4 equal groups of 40 birds each. The 12 days old chickens from the group I were orally infected with 8.10⁴ sporulated *E. tenella* oocysts. In the group II, chickens were inoculated with *E. coli O78* 1.10⁷ CFU/mL via single intraperitoneal injection (1 mL) at the age of 16 days [29]. Birds from the group III were infected with *E. tenella* oocysts at the same age and route as described for the group I then, 4 days later (i.e. when they were 16 days old), they were challenged with *E. coli* in the same conditions than for the group II. The birds of the group IV were neither infected nor challenged and served as negative controls.

At the 8th day after *E. tenella* infection or the 4th day after *E. coli* challenge, blood samples were collected from *v. ulnaris superficialis* into sterile tubes with lithium heparin as anticoagulant. After centrifugation (750g, 4°C, 15 minutes), plasmas were carefully harvested and stored at -20°C until biochemical analysis. Thereafter, chickens were slaughtered by cervical dislocation and internal organs were rapidly removed (within one hour after death).

**Materials and Methods**

**PREPARATION OF THE PATHOGEN AGENTS**

*E. tenella* oocysts were obtained from chickens naturally infected in a spontaneous field outbreak. For enrichment, oocysts were passed twice through 2-week-old chickens, allowed to sporulated and stored by means of routine techniques. Sporulated oocysts were administered by means of ingluvil tube. Oocysts used for infection were tested for sterility in both MacConkey and blood agars [41].

On the other hand, a field *E. coli* strain, isolated with poultry with septicaemia, serotyped as O78, was used.

**ANIMALS AND PROTOCOL DESIGN**

One hundred and sixty broiler chickens, Cobb 500, were used in the experiment. The birds were obtained from a hatchery, free of infectious diseases. Five days prior to the experimental infection, chickens were investigated for occurrence of pathogen *E. coli* strains by the method described by NAGI and MATHEY [37]. Chickens were reared in cages on slatted floors in a premise under ambient temperature of 25°C, normal light regimen, fed with compound food according to their age without antibiotics and coccidiostatics. The rearing conditions minimizd the possibility for infection with either *Eimeria* or pathogen *E. coli* (EPEC).

Kidneys, liver, heart, Fabricius bursa, spleen, intestine, brain and cerebellum were fixed in 10% neutral formalin for histopathological investigations. Fixed material was embedded in paraffin and 6-μm cross sections were stained with haematoxylin-eosin. PAS reaction was used to detect lipoproteins, glycoproteins and mucoproteins in various tissues and cell components as well as for determination of the thickness of main tubular membranes with lipoprotein structure [10].
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Plasma ASAT and ALAT activities were assayed by methods described by Popov and Dishlyanova [40] whereas the ALP activities were determined according to the method of BESSEY *et al.* [3].

**STATISTICAL ANALYSIS**

Data were statistically processed by one-way ANOVA with LSD as post-hoc test. The results are presented as means ± standard errors of means (SEM). The level of significance was set at *P* < 0.05.

**Results**

**GROSS ANATOMICAL AND HISTOPATHOLOGICAL FINDINGS**

The table I summarized the pathological lesions induced by exposure to *E. tenella* and/or *E. coli*. No lesion was recorded in negative controls at the macroscopic and microscopic levels.

In chickens infected with *E. tenella* (groups I and III), primary gross anatomical changes were observed in caecum which was enlarged and filled with bloody content whereas the mucous caecal coat was scattered with haemorrhages (figures 1 and 2). Some dystrophic processes in the lining and glandular epithelia, local mononuclear proliferation and oedema of *propria* were also evidenced by histology. No lesion was noted in caecum from birds exposed only to *E. coli* (group II).

Often, the liver of birds challenged with *E. tenella* alone exhibited a grey-yellowish staining of a large part of surface, hyperaemia, dystrophic and granular degeneration in hepatocytes (figure 3). A fibrinous pericapsulitis with a slight green to a bronze appearance of the liver was also noticed in birds co-exposed to *E. tenella* and to *E. coli* (group III) (figures 2 and 4) or infected only with colibacteria (group II). In these 2 groups, the liver sinusoid capillaries were strongly hyperaemic (figure 4): some necrotic foci impregnated with fibrin were observed in some areas and small fibrin thrombi were trapped in smaller vessels or capillaries. Furthermore, enlargement and oedema of gallbladder were also observed in the both groups inoculated with *E. coli* (groups II and III).

In all chickens exposed to pathogens, kidneys exhibited granular dystrophy in the epithelium of proximal tubules (figure 5). Strong hyperaemia of peritubular capillaries and small fibrin thrombi were seen in some vessels or were focally deposited onto glomeruli only in chickens infected with *E. coli* (groups II and III). Fibrinous casts were also seen in tubular lumens of the kidneys in the chicks of the same groups (figure 6).

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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<tr>
<td>Caecum</td>
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<tr>
<td>Bloody content</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
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<td>Haemorrhages, oedema</td>
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<td>-</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Fibrinous perihepatitis</td>
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<td>+++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Hyperaemia of sinusoid capillaries</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Granular degeneration in hepatocytes</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
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<td>Fibrin thrombi in the vessels</td>
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<td>+++</td>
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<td>Oedema of gallbladder</td>
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<td>Kidney</td>
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<tr>
<td>Granular degeneration</td>
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<td>++</td>
<td>+++</td>
<td>-</td>
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<td>Hyperaemia, fibrin thrombi</td>
<td>-</td>
<td>++</td>
<td>++</td>
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<td>Fibrinous casts in tubules</td>
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<td>++</td>
<td>-</td>
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<tr>
<td>Heart</td>
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<tr>
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<td>-</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Intermuscular oedema, fibre lysis</td>
<td>++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Air-saccule</td>
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<tr>
<td>Fibrinous air-sacculitis</td>
<td>-</td>
<td>+++</td>
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<tr>
<td>Brain, cerebellum</td>
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<tr>
<td>Perivascular/pericellular oedema</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
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<td>Degeneration in glial cells</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
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<td>Tyrolysis or pyknosis in neurons</td>
<td>+</td>
<td>-</td>
<td>++</td>
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</table>

*: no change; +: slight changes; ++: moderate changes, +++: severe changes.

Table I: Lesions induced in chickens 8 days after *E. tenella* invasion or 4 days after *E. coli* infection. Group I: chickens (12 days old) orally infected with *E. tenella* oocysts (8.10⁴), group II: chickens (16 days old) intraperitoneally infected with *E. coli* O78 serotype (10⁷ CFU), group III: chickens (12 days old) co-infected with *E. tenella* and 4 days later with *E. coli*, group IV: negative controls (n = 10 in each group).
Fibrinous pericarditis (figure 2) and fibrinous air-sacculitis were observed in most of the chickens infected with *E. coli*. Small haemorrhages were also noticed on the pericardium and proventricular serous coat in chickens from the same both groups II and III. In the myocardium, perivascular and intermuscular oedema in addition to some lytic changes in muscle fibres were seen at different degrees in all treated chickens (figure 7).

Clinically, limb paresis was frequently observed in chickens co-treated with *E. tenella* and *E. coli*. Perivascular and pericellular oedema were observed in brains of chickens exposed to *E. tenella* alone or coupled to *E. coli* inoculation. Slight to moderate ballooned degeneration in the glial cells, together with slight tigrolysis or pyknosis in some neurones were found in the brains of all *E. tenella* exposed chickens. In the cerebellum of the same chickens, lytic processes in the region of Purkinje cells were often found in addition to pericapillary oedema in the granular or molecular layer, (figure 8).

**BIOCHEMICAL FINDINGS**

As shown in Table II, plasma activities of the 3 studied enzymes (ASAT, ALAT and ALP) were significantly (*P* < 0.05) modified compared to negative controls 8 days after *E. tenella* invasion or 4 days after *coli*-infection. Significant increases of the activities of the 2 transaminases were evidenced in all treated birds; the highest elevations of the enzyme activities were recorded in chickens exposed to the 2 pathogens (group III), intermediate changes were obtained in the group II (only *coli*-infection) and the lowest increases were observed in the group only infected with *E. tenella* (group I) (1 vs. III: *P* < 0.05, for the ALAT activity). By contrast, the alkaline phosphatase activity varied in the opposite way. Compared to negative controls, this enzyme activity was significantly depressed in birds infected with *E. coli* alone or combined with *E. tenella* invasion (*P* < 0.05) and the maximal decrease was recorded for the group III (1 vs. III: *P* < 0.05).

In our experiments, chickens infected only with *E. coli*, as well as co-infected with *E. coli* and *E. tenella*, showed degenerative and circulatory alteration in kidneys, enlarged, hyperaemic and green-tinted liver, oedema and enlargement of the gallbladder as well as fibrinous air-sacculitis, pericarditis and perihepatitis. These changes together with the haemorrhagic inflammation of caecum showed that both infections (parasitic and bacterial) occurred simultaneously, as early as 24-48 hours after *E. coli* challenge. Chickens challenged with both agents (*E. coli* and *E. tenella*) showed more severe clinical, biochemical and pathoanatomical findings. In the third experimental group, limb paresis was even observed followed with a rapid lethal issue. This fact evidenced the synergism between both factors, resulting in a more critical pathological. In this group, brain alterations were more marked and probably induced pareses. According to most investigators, small chickens or turkeys dead from acute septicaemia usually showed enlarged and green-tinted liver, hyperaemic spleen, oedematous gallbladder and some fluid accumulated in body cavities, less frequently serofibrinous exudate on air sacs and liver and heart serous coats [29]. Apart the described changes in our experiment with *E. coli*–infected chickens, we observed also a strong enlargement of gallbladder, oedema of its wall, more pronounced fibrinous air-sacculitis, pericarditis and perihepatitis, haemorrhages on the serous coat of the proventriculus and multiple fibrin thrombi in blood vessels that are more rarely reported in available literature. The described pathoanatomical changes in this experiment were present in chickens infected only with *E. coli*, as well as in birds, co-infected with *E. coli* and *E. tenella*, thus showing that both the parasitic and the bacterial infections occurred in parallel. The fibrinous pericarditis, perihepatitis and air-sacculitis, as well as gallbladder enlargement and gall bladder wall oedema in adult chickens were also reported by other authors as a subacute syndrome of colibacteriosis with reduced immunity [2, 24, 30, 33, 42].

The gross anatomical and histopathological findings in the liver were also similar to those previously reported [2, 5, 6, 33, 38, 39, 45], but in the present experiment, multiple fibrin thrombi in blood vessels that are less frequently reported were also observed. Another interesting fact is the occurrence of unusually intensive degenerative changes in kidneys, including the fibrin thrombi in capillaries in chickens infected only with *E. coli* and particularly in those co-infected with *E. coli* and *E. tenella*. Some biochemical alterations (increased concentrations of urea, creatinine and uric acid) indicating kidney failure, were also reported in previous investigations.

### Table II: Plasma ASAT, ALAT and AP activities in chickens 8 days after *E. tenella* invasion or 4 days after *coli* infection. Group I: chickens (12 days old) orally infected with *E. tenella* oocysts (8.10⁴), group II: chickens (16 days old) intraperitoneally infected with *E. coli* O78 serotype (10⁷ CFU), group III: chickens (12 days old) co-infected with *E. tenella* and 4 days later with *E. coli*, group IV: negative controls. Results are expressed as mean ± SEM (n = 10 in each group).

<table>
<thead>
<tr>
<th>Group</th>
<th>ASAT (U/L)</th>
<th>ALAT (U/L)</th>
<th>ALP (mmol/h/L)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>25.24 ± 1.26b</td>
<td>10.60 ± 0.48b</td>
<td>0.218 ± 0.003ab</td>
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<tr>
<td>II</td>
<td>27.60 ± 7.70b</td>
<td>12.80 ± 0.92bc</td>
<td>0.203 ± 0.010bc</td>
</tr>
<tr>
<td>III</td>
<td>37.94 ± 3.40bc</td>
<td>15.11 ± 0.52c</td>
<td>0.165 ± 0.010c</td>
</tr>
<tr>
<td>IV</td>
<td>11.34 ± 3.23a</td>
<td>4.08 ± 0.52a</td>
<td>0.256 ± 0.020a</td>
</tr>
</tbody>
</table>

ASAT: Aspartate aminotransferase; ALAT: alanine aminotransferase; AP: Alkaline phosphatase; Different superscripts a,b,c in the same row indicate significant difference (*P* < 0.05) between groups.

**Discussion**

In our experiments, chickens infected only with *E. coli*, as well as co-infected with *E. coli* and *E. tenella*, showed degenerative and circulatory alteration in kidneys, enlarged, hyperaemic and green-tinted liver, oedema and enlargement of the gallbladder as well as fibrinous air-sacculitis, pericarditis and perihepatitis. The described pathoanatomical changes in this experiment were present in chickens infected only with *E. coli*, as well as in birds, co-infected with *E. coli* and *E. tenella*, thus showing that both the parasitic and the bacterial infections occurred in parallel. The fibrinous pericarditis, perihepatitis and air-sacculitis, as well as gallbladder enlargement and gall bladder wall oedema in adult chickens were also reported by other authors as a subacute syndrome of colibacteriosis with reduced immunity [2, 24, 30, 33, 42].
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**Figure 1**: Enlarged caecum filled with bloody content (a) in a chick exposed to *E. tenella* (8 days after the challenge).

**Figure 2**: Enlarged caecum filled with bloody content (a), fibrinous pericarditis (b) and slight perihepatitis (c) observed in a chick co-exposed to *E. tenella* and *E. coli* (4 days after the bacterial challenge).

**Figure 3**: Granular degeneration in hepatocytes (a) in liver of chick exposed to *E. tenella* alone. H/E X260.

**Figure 4**: Fibrinous perihepatitis (a), granular degeneration (b) and strong hyperaemia of sinusoid capillaries (c) in liver of chick exposed to *E. tenella* and *E. coli* both. H/E X300.

**Figure 5**: Granular degeneration in the epithelium of proximal tubules (a) of kidney in chick exposed to *E. tenella* alone. Haematoxylin-eosin X300.

**Figure 6**: Fibrinous casts in tubular lumens (a) and hyperaemia of peritubular capillaries (b) in kidney in chick infected with *E. coli* alone. Haematoxylin-eosin X200.
on infected turkeys by KOYNARSKI [26] albeit kidney damage are not generally reported. It should be also mentioned that haemorrhages on the proventricular serous coat observed in both groups challenged with \textit{E. coli}, are not reported in available references. The thrombotic events in small blood vessels and the impaired permeability of vessels (leakage of significant amount of fibrin outside and the multiple haemorrhages) in co-infected birds, resulting in additional impairment of tissue circulation in many organs and causing more severe pathological alterations including necrosis, appeared to be very intense and systemic. The more significant changes in kidneys and liver in the group treated with both \textit{E. coli} and \textit{E. tenella} showed that apart the dystrophy caused by \textit{E. coli}, a considerably more marked dystrophy occurred in these organs because of their participation in the elimination of toxic products secondary to the \textit{E. tenella} development in caecum.

The blood biochemical analysis reflected the functional organ alterations [44] and some enzyme activities are often used as indicators of the site and of the extent of pathological injury [8]. With this regard, their use in organ-specific diagnostics is advised [20]. It is known that apart organ-related specificity, enzymes could indicate injury in several cell organelles. Thus, ALAT is localized only in the cytosol, whereas ASAT is localized both in cytosol (about 40% as ASAT1) and in mitochondria (ASAT2) [15], therefore the determination of its plasma activities provides information not only for hepatocyte damage, but for the severity of the process as well. Alkaline phosphatase (ALP) is localized in cell membrane [27].

In the present study, in birds infected with \textit{E. tenella} or/and \textit{E. coli}, statistically significant differences occurred between challenged and healthy chickens with regard to ALP, ASAT and ALAT activities. By studying blood serum and organ ALP activity in chickens, GORANOV [14] evidenced that as this enzyme was most abundant in the duodenum, liver and proventriculus, deviations in its activities indicate some damage in these organs. In experimental renal damage, reduced activities of ALP and cholinesterase were reported [12].

Reduction in ALP was observed in turkey hens infected with \textit{E. adenoeides} [26]. The decrease in ALP in \textit{E. tenella} infected chickens was interpreted as degenerative changes in the intestinal wall by MUSAEV \textit{et al.} [36].

Electron microscopy of liver in \textit{E. tenella} infected chickens [32] showed damage in both mitochondrial membranes and cytoplasm in liver cells. Consequently, elevated ASAT activities could be due to dystrophic and necrotic processes in liver and heart, observed in treated birds. As it is known that more than 50% of ASAT activity is related to these cellular alterations, these events contributed to statistically significantly higher blood enzyme activities in all experimental groups and especially in co-infected birds.

Alanine aminotransferase is a liver-specific enzyme [21, 27]. The changes in ALAT concentrations are mainly related to dystrophic alterations in liver and leakage through ruptured membranes. In bacterial infections such as acute fowl typhoid, blood ASAT and ALAT were increased in the first days of the experimental infection [26]. In chickens, infected with \textit{Eimeria}, a statistically significant increase in ASAT and a weak elevation of ALAT was reported [19]. Higher ASAT and ALAT activities with unchanged AP values are communicated in turkey hens and chickens with experimental colibacillosis [28, 33]. The more pronounced changes in the values of these parameters in co-infected chickens (\textit{E. tenella} + \textit{E. coli}) observed in this experiment, are highly probably related to the more severe pathological processes.

As a conclusion, the present experiment shows that pre-invasion with \textit{Eimeria tenella} strongly aggravates colibacillosis and suggests that, practically, similar mixed bacterial and parasitic infections can be frequently observed, further complicating the pathoanatomical and biochemical findings and impeding the proper diagnostics. That is the reason why the knowledge of the more complex clinical and morphological findings in such cases is very useful with regard to the timely diagnostic and curative and preventive measures against both disease agents – the parasitic and the bacterial ones.
References


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