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Effect of experimentally induced aflatoxicosis on haematological parameters and bone marrow morphology in mulard ducks

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Abstract. In this experiment, the toxic effects of AFB₁ on some haematological parameters were investigated in mulard ducks, namely red blood cell counts (RBC), haemoglobin content (HGB), haematocrit (HCT), platelet counts (PLT), white blood cell counts (WBC), differential white blood cell counts (WBC %), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Experiments were conducted with 4 groups of 20 10-day-old mulard ducks each. The groups were as followed: group I – control, fed a standard compound feed according to the Department of General and Clinical Pathology, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

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Keywords: aflatoxin B₁, haematological parameters, bone marrow, mallard ducklings Mycotox Ng

Introduction

Mycotoxins are compounds with various chemical structure and biological effects produced by filamentous fungi (Sudak, 2003). Aflatoxins, ochratoxins, trichotheccene mycotoxins, zearalenone and fumonisins are the most important for human health and animal productivity (Zain, 2011). Mycotoxicoses is a collective term comprising diseases in animals and humans produced by ingestion of mycotoxins (Hussein and Brasel, 2001). Exposure of farm animals to toxigenic fungi and their toxins occurs mainly by ingestion of contaminated feeds which contain corn, peanut or sorghum (Kamei and Watanabe, 2005; Tucz et al., 2010). Aflatoxins are secondary toxic metabolites of fungi from the genus Aspergillus (A. flavus, A. parasiticus and A. nomius) (Kurtzman et al., 1987). They belong to the group of storehouse moulds. Under field conditions, before harvesting and storage of cereal crops, growth of moulds from the genus Fusarium (Gramineaeum, Culmorum, Claviceps purpurea) producing zearalenone, fumonisins and trichotheccene mycotoxins is more common, while during storage of feeds, fungi from genera Aspergillus and Penicillium (Aspergillus flavus, Aspergillus parasiticus and Penicillium verrucosum) producing aflatoxins (B₁, B₂, G₁, G₂) and ochratoxins occur (Hashmi et al., 2006). The temperature range within which A. flavus and A. parasiticus produce aflatoxins is from 12 to 41°C, with optimum range 25–32°C (Lillehoj, 1983), air humidity higher than 62% and feed humidity higher than 14% (Royes and Yanong, 2002). Aflatoxins are fluorescence compounds. Aflatoxin B₁ (AFB₁) and aflatoxin B₂ (AFB₂) emit blue fluorescence, while aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂) emit green fluorescence after UV irradiation (Hussein and Brasel, 2001). Aflatoxins are highly toxic for men and animals and among the most potent natural poisons. Similarly to the other mycotoxins, they are thermostable (Cheeke, 1998). The EC legislation as well as that of Chile, Canada and the USA allows no more than 10 ppb AFB₁ in poultry feeds and 20 ppb of total aflatoxin (AFB₁ + AFG₁ + AFB₂ + AFG₂) in feeds for the poultry industry (Directive 2002/32/EC; Fonseca, 2003). More than 18 different aflatoxins are identified (B₁, B₂, G₁, G₂, P. Q. M., M., B.a etc.). The commonest aflatoxins with toxicological importance are produced by moulds growing on feeds under natural conditions - B₁, B₂, G₁, and G₂; and M. and M. in milk (IARC, 2002). Aflatoxin B₁ (AFB₁) is the most toxic metabolite for men, animals and hydrobionts (Kennedy et al., 1998). It is a potent natural hepatotoxin, carcinogen, teratogen and mutagen (Han et al., 2008). Terao and Ueno (1978) reported that the toxicity of AFG₁, AFB₁ and AFG₂ was equal to 10%, 20% and 50% respectively of that of AFB₁. Aflatoxins are found to contaminate naturally mainly cereal and oily crops, nuts and milk (Wu et al., 2009). On the other hand, 77% of naturally occurring aflatoxins is from the B type (Wilson and Payne, 1994). Aflatoxins are a potential threat to poultry health and performance after consumption of contaminated fodders (Huff et al., 1986). From all fowl species, growing ducklings, goslings, turkey poult and chicks are more susceptible to the toxic effect of aflatoxins. The latter are mainly localised in the liver and are characterised with necrosis, haemorrhages and proliferation of biliary duct epithelium. Chronic aflatoxicosis in this bird species results in weight loss, reduced feed conversion, increased sensitivity to infections and lower egg production (Dalvi, 1986). In an experiment with broiler chickens, Al-Daraji et al. (2004) demonstrated changes in haematological and blood biochemical parameters (red and white blood cell counts, haematoconit, haemoglobin, heterophil and lymphocyte percentages, uric acid, blood glucose, cholesterol, total protein, calcium phosphate, gamma-glutamyltransferase and alkaline phosphatase
activities.

One of the most appropriate methods for detoxication of mycotoxin-contaminated feeds is the use of non-nutritional adsorbents that are capable to bind toxins and to inhibit their absorption from the gastrointestinal tract, and thus, to minimise their toxic effects on animals and their occurrence in animal organs and animal foodstuffs (Ramos et al., 1996; Bintvihot et al., 2002; Kana et al., 2011; Oguz, 2012). A good mycosorbent should be able to restore the nutritional value of aflatoxin-contaminated feeds. The quality of mycosorbents is assessed by four principal parameters, namely: binding capacity, absorption efficiency, activation time and binding extent (Van Kessel and Hiang-Chek, 2004). The process of aflatoxin binding by mycosorbents is based on the electric polarity principle. The negative electrical charge of the mycotoxin binds to the positive charge of the mycosorbent and thus, mycotoxin are immobilised and subsequently, removed from the animal body. The effects of aluminiumsilicates, active charcoal and yeasts are extensively studied, and promising results have been reported (Ramos et al., 1996; Huwig et al., 2001; Mabbet, 2005; Girish and Devegowda, 2006; Oguz, 2012).

The aim of the present study was to monitor the changes in some haematological parameters in mulard ducks with experimental aflatoxicosis. Also, the purpose was to evaluate the possibility for prevention of toxic effects of AFB, by feed supplementation with the mycosorbent Mycotox NG.

Material and methods

Experiments were conducted with 80 female 10-day-old mulard ducks, divided into 4 groups according to the following design:

- Group I – control. It was fed a standard compound feed according to the age produced by Zoohrinvest feed factory, Stara Zagora and comprised pelleted starter, grower and finisher;
- Group II – experimental, the standard feed was supplemented with 0.5 mg/kg aflatoxin B1;
- Group III – experimental, the standard feed was supplemented with 0.8 mg/kg aflatoxin B1;
- Group IV – experimental, the standard feed was supplemented with 0.5 mg/kg aflatoxin B1 and 2 g/kg Mycotox NG (Ceva Sante Animale, France).

The aflatoxin B1, used in this experiment was produced by Aspergillus flavus (99% purity) purchased from Sigma-Aldrich, Germany. The feed of experimental groups of birds was grinded for better homogenisation of aflatoxin B1. Microclimatic parameters were optimal and equal for all groups. In the beginning of the trial, air temperature in the living area of ducks was 35°C and until the 15th day it decreased at a daily rate of 1°C; by day 28 it was 20°C, and thereafter – +18°C with air humidity of 60–75% (Regulation No. 44/20.04.2006). The duration of the light day was 24 hours throughout the experiment. Control and experimental groups were housed in different sections, each area with 4 M4 in the same premise. The sections were bedded with clean dry wooden shavings with layer depth of 5cm. During the first week of life, the feeding width was 1cm and subsequently: 10cm. Blood samples were collected from v. metatarsalis medialis on experimental days 21 and 42 in sterile vacutainers with K3EDTA (FL medical, Italy) for determination of: haemoglobin content (colorimetric test, Human Diagnostica Germany), haematocrit (microcentrifugation method, Angelov et al., 1999), red blood cell counts, platelet counts, white blood cell counts (counted in a chamber as per Angelov et al., 1999). Differential white blood cell counts (WBC, %), were determined on stained blood smears. The experiment was approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine to the Trakia University (permit No. 42.10.10.2011).

The statistical analysis of data was done by one-way ANOVA, and the level of statistical significance – with the Tukey-Kramer test (p<0.05).

Results

Blood picture

The parameters of the red blood picture (erythrocytes, haemoglobin, haematocrit, platelets) in control and experimental groups are presented in Table 1. Red blood cell counts in Groups II and III were statistically significantly lower at both sampling days (21 and 42) compared to control birds (p<0.001). Their values on day 21 were 2.52±0.05 T/l (17.92%) and 2.34±0.05 T/l (23.78%) vs control group (3.07±0.07 T/l) and by the 42nd day the changes were more pronounced with 2.31±0.02 T/l (21.96%) in experimental group II and 2.10±0.05 T/l (29.06%) in experimental group III (control group – 2.96±0.06 T/l). Haemoglobin contents were statistically significantly lower in groups II and III than in untreated birds (p<0.001): on the 21st day, control birds’ haemoglobin was 137.57±1.27 g/l vs 117.63±1.10 g/l (14.5%) in group II and 111.53±1.35 g/l (18.93%) in Group III while on day 42, the values in challenged groups were 107.76±2.60 g/l (20.18%) and 96.9±3.85 g/l (28.18 %) in groups II and III, respectively (control group had average haemoglobin of 135.0±1.43 g/l). In ducklings treated only with AFB, (Groups II, III) haematocrit decreased significantly on both sampling days (p<0.001). On day 21, haematocrit in Group II was 32.4±1.23% (18.39 %), and of Group III: 28.9±0.79% (27.21%) than in controls (39.7±0.81%). On day 42, haematocrit in Group II was 29.6±1.07% (22.92%) and in Group III: 24.9±0.95% (35.16%) which was substantially lower than control values (38.4±0.76%; p<0.001). Similar changes were observed in platelet counts – on the 21st day, average platelet counts were 60.69±2.99 G/l (26.96 %) in Group II and 44.29±2.80 G/l (46.7%) in Group III vs controls (83.09±2.93 G/l, p<0.001). At the next sampling day, changes were even more pronounced with average platelet counts of 55.00±1.75 G/l (33.95%) and 40.56±1.48 G/l (51.29%) in Groups II and III, respectively (p<0.001). Control platelets at day 42 were 83.26±6.38 G/l. In Group IV, the changes in these red blood cell parameters were reduced at a various extent compared to controls (p<0.50; p<0.01). Red blood cell counts on days 21 and 42 were 2.80±0.06 T/l (8.80%) and 2.77±0.04 T/l (6.42%) respectively. Haemoglobin content was 130.85±1.64 g/l (4.89%) on day 21 and 124.49±2.47 g/l (7.79%) on day 42. On day 21, average haematocrit was 36.2±0.72% (8.81%), and on day 42: 34.9±0.73 % (9.12%). Platelet counts on days 21 and 42 were 71.74±2.07 G/l (13.66%) and 88.61±2.23 G/l (17.6%) respectively.

Table 2 presents the data about white blood cell counts (total and differential) in untreated birds and those fed aflatoxin with the feed. Total white blood cell counts (WBC) was statistically significantly increased in Groups II and III than in controls. In Group II, WBC were 28.08±0.79 G/l (37.57%) and 32.95±0.98 G/l (47.16%) on days 21 and 42, respectively (p<0.001). In Group III, WBC on day 21 was 31.85±0.76 G/l (56.05%) whereas on day 42 - 38.25±1.18 G/l (70.83%) (p<0.001). The addition of mycosorbent to the contaminated feed (Group IV) partly reduced the deleterious effects of AFB, on total WBC counts which were 24.32±1.07 G/l (19.15%) on day 21 and 27.69±1.74 G/l (23.67%) on day 42 (p<0.05).
### Table 1. Changes in haematological parameters (red blood cell counts, haemoglobin, haematocrit, platelet counts) in mulard ducks receiving aflatoxin B₁ (AFB₁) either alone or together with Mycotoxin NG

<table>
<thead>
<tr>
<th>Groups</th>
<th>Red blood cell counts, T/l</th>
<th>Haemoglobin, g/l</th>
<th>Haematocrit, %</th>
<th>Platelet counts, G/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 21</td>
<td>Day 42</td>
<td>Day 21</td>
<td>Day 42</td>
</tr>
<tr>
<td>I</td>
<td>3.07±0.07</td>
<td>2.96±0.07</td>
<td>137.57±1.27</td>
<td>135.00±0.81</td>
</tr>
<tr>
<td>II</td>
<td>2.52±0.05</td>
<td>2.31±0.05</td>
<td>117.63±1.10</td>
<td>107.76±1.23</td>
</tr>
<tr>
<td>III</td>
<td>3.41±0.05</td>
<td>2.10±0.05</td>
<td>111.53±1.02</td>
<td>96.96±1.13</td>
</tr>
<tr>
<td>IV</td>
<td>2.80±0.05</td>
<td>2.77±0.05</td>
<td>130.55±1.64</td>
<td>124.49±1.75</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM; n=20 in each group; P<0.05; P<0.01; P<0.001; 1 vs control group; 2 vs experimental group I; 3 vs experimental group II.

The differential white blood cell counts exhibited statistically significant changes in heterophils and lymphocytes (Table 2). Heterophil percentages were very increased in Groups II and III on day 21 - 41.6±0.93% (38.66%) and 45.1±1.29% (50.33%), as well as on day 42 - 44.5±1.00% (44.62%) and 48.1±1.13% (54.16%) (p<0.001) compared to control group (30.00±1.57%; 31.2±1.81%). More pronounced changes were established on day 42. In Group IV, heterophil percentage was somewhat lower after the addition of mycosemb (p<0.05): 36.2±1.57% on day 21 and 37.1±1.44% on day 42. The reduction vs control group was by 20.66% and 18.91% respectively. Lymphocyte percentage on day 21 was 51.00±1.47% (18.14%) in Group II and 47.2±1.34% (24.24%) on Group III (p<0.001). On the 42nd day, respective values in Group II and III were 48.0±0.02% (21.70%) and 45.0±0.01% (26.60%) in Group II (p<0.001). In Group IV, changes were less manifested after the addition of the mycosorbent (p<0.05) with values by day 21 of 57.1±1.15%, and by day 42 of 55.6±1.19%. The reduction was by 8.34% and 9.30%, respectively. Statistically significant changes between monocytes, eosinophils and basophils in untreated and experimental ducks were not found out (p<0.05).

There were no statistically significant changes in mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) between control and treated mulard ducks (Table 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>White blood cell, G/l</th>
<th>Heterophil Le, %</th>
<th>Eosinophil Le, %</th>
<th>Lymphocyte, %</th>
<th>Monocyte, %</th>
<th>Basophil Le, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 21</td>
<td>Day 42</td>
<td>Day 21</td>
<td>Day 42</td>
<td>Day 21</td>
<td>Day 42</td>
</tr>
<tr>
<td>I</td>
<td>20.41±1.04</td>
<td>22.39±1.19</td>
<td>30.00±1.57</td>
<td>31.2±1.61</td>
<td>2.70±0.36</td>
<td>2.60±0.13</td>
</tr>
<tr>
<td>II</td>
<td>28.08±0.97</td>
<td>32.95±1.57</td>
<td>41.3±1.81</td>
<td>44.5±2.30</td>
<td>2.30±0.21</td>
<td>2.40±0.33</td>
</tr>
<tr>
<td>III</td>
<td>31.85±1.76</td>
<td>38.25±1.96</td>
<td>45.1±1.78</td>
<td>48.1±2.50</td>
<td>2.50±0.22</td>
<td>2.40±0.33</td>
</tr>
<tr>
<td>IV</td>
<td>24.32±1.07</td>
<td>27.69±1.74</td>
<td>36.2±1.74</td>
<td>37.1±1.87</td>
<td>2.40±0.45</td>
<td>2.20±0.50</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM; n=20 in each group; P<0.05; P<0.01; P<0.001; 1 vs control group; 2 vs experimental group I; 3 vs experimental group II.

### Discussion

Pathomorphological studies
The bone marrow of ducklings from Groups II and III exhibited hypocellularity, reduced number of haematopoietic stem cells and initial extent of fatty infiltration (Figure 1). These events were more pronounced in birds from Group III (Figure 2). In birds from Group IV, bone marrow hypocellularity was significantly less pronounced than that of Groups II and III (Figure 3). Histopathological changes in the bone marrow of control birds were not observed.

Aflatoxins, their metabolite and reactive oxygen species (ROS) produced by them impair haematopoiesis in bone marrow and lymphatic organs, where blood cells and other blood components are formed (Halliwell, 2007). Aflatoxins induce haematological disturbances (Dietert et al., 1983). They lead to haematopoietic suppression and anaemia by reduction of red blood cells, mean corpuscular volume and haemoglobin (Reddy and Waliyar, 2012).
It is reported that aflatoxins cause leuco- and monocytopena and at the same time, increase neutrophil percentage in blood (Dommez et al., 2012). In cattle, blood coagulation disorders with prolonged prothrombin time has been observed (Thrasher, 2012).

Reduced haematocrit, haemoglobin, erythrocytes and lymphocyte percentage was observed in broiler chickens with experimental aflatoxicosis, probably due to the inhibition effect of aflatoxins on haematopoietic organs (Campbell et al., 1983; Huff et al., 1986, 1988; Kubena et al., 1990; Kececi et al., 1995, 1998; Oguz et al., 2000; Sakhare et al., 2007; Mohamed and Mohamed, 2009). Similar changes were observed in this study as well. On the other hand, this decrease in red blood parameters results from inhibition of protein synthesis (Kubena et al., 1993; Abdel-Wahhab et al., 2002) or the faster degradation of erythrocytes in the spleen (Mokif and Muiz, 2015). It was found out that aflatoxin B, decreases iron-binding capacity of erythrocytes (Harvey et al., 1991) and impairs the metabolism of some trace elements (Cu, Zn) involved in haematopoesis (Abdel-Wahhab, et al., 2002). Reduced red blood cell counts and mean corpuscular volume is reported in broiler chickens fed feeds contaminated with aflatoxins (Singh et al., 1992), ochratoxins (Doerr and Huff, 1980; Aved et al., 1991), or combination of the two mycotoxins (Sawarkar et al., 2011). Similar changes in red blood cell counts and mean corpuscular volume were found out in fish fed feeds containing aflatoxins (El-Bouhy et al., 1993). The observed anaemia is secondary to the lower rate of DNA and protein synthesis and is enhanced by the deficiency of B, and folic acid (Tessari et al., 2006). Lower values of haematological parameters were probably due to protein synthesis inhibition (Kaneko et al., 1989), lower iron-binding capacity (Harvey et al., 1991) or impaired function of haematopoietic organs (Van Vleet et al., 1992; Abdel-Wahhab et al., 2002). Observed oligochromatemia could be explained with the small size of erythrocytes, impaired blood synthesis in bone marrow or lower rate of red blood cells formation (Sharma et al., 2011). Impaired haematogenesis could be also attributed to aflatoxin-induced oxidative stress (Owen et al., 2000).

Increased white blood cell counts and heterophil percentages are presumably due to the irritating effect of aflatoxins on gastrointestinal mucosa and its inflammation (El-Lethy and El-Zorb, 2004; Sakhare et al., 2007; Safamehr, 2008). Observed lymphocytopaenia could be attributed to the toxic effects of aflatoxins on cells in the peripheral circulation or suppression of bone marrow and lymphoid organs function (Oguz et al., 2000; Mohamed and Mohamed, 2009). Increased white blood cell counts and heterophil percentages could be also explained with bone marrow changes (Abdel-Wahhab et al., 2002).

**Table 3.** Changes in mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) in mulard ducks receiving aflatoxin B, (AFB, ) either alone or together with Mycotox NG

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCV, pg</th>
<th>MCHC, g/l</th>
<th>MCH, f/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 21</td>
<td>Day 21</td>
<td>Day 21</td>
</tr>
<tr>
<td></td>
<td>129.58±4.00</td>
<td>383.00±9.47</td>
<td>35.3±6.82</td>
</tr>
<tr>
<td></td>
<td>128.57±4.10</td>
<td>388.56±11.89</td>
<td>39.4±20.31</td>
</tr>
<tr>
<td></td>
<td>118.59±4.40</td>
<td>387.33±9.47</td>
<td>35.3±6.82</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SEM; n=20 in each group; P<0.05; P<0.01; P<0.001; 1 vs control group; 2 vs experimental group I; 3 vs experimental group II*
Increase in the heterophil percentages might indicate a tendency of the organism to compensate for the decrease of its resistance (Sova et al., 1991). In mice treated with T-2 toxin, Sinozuka et al. (1998) reported decreased cell density in the haemopoitetic parenchyma. The changes were observed in the metaphysis and diaphysis regions. Hypocellularity was a result from reduction of myelocytes, immature granulocytes, erythroblasts and lymphocytes. Myelocytes exhibited karyorhexis, karyopyknosis, fragmentation and neutrophils engulfed by adjacent macrophages. In megakaryocytes, nuclei with irregular size, increased number of nuclei and intracellular cells were found out. Bone marrow and spleen red pulp hypoplasia were reported in mice challenged with T-2 toxin (Smith et al., 1994).

Contrary to the present results, Aved et al. (1991), Mohiuddin et al. (1993) and Mohamed and Mohamed (2009), reported lower white blood cell counts in broiler chickens that received feed contaminated with aflatoxins and ochratoxins. Decrease blood cell counts is a reflects deleterious effects of AFB, on haemopoitetic tissue (Mohiuddin et al., 1993) and immune systems (Oguz, 1997). Furthermore the variation in the hematological values may be due to the temperature variations, weather changes, dose levels, geographical distribution or age related factors (Mahmood et al., 2017).

The lack of statistically significant changes in eosinophils, monocytes and basophils observed in this study agreed with other reported results (Oguz et al., 2000; Mohamed and Mohamed, 2009). Similarly, the established lack of changes in mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) in mulard ducks were in line with the previous results of other authors (Oguz et al., 2000; Basmacioglu et al., 2005). The utilisation of mycosorbents is one of the most reliable methods for reduction of the toxic effect of mycotoxins on animals (Dakovic et al., 2005). Adsorbents have a high affinity to aflatoxin binding in the animal gastrointestinal tract and thus, they decrease their absorption and hence, deleterious effect on birds (Raju and Dewegowda, 2000; Eraslan et al., 2005). Previously, the harmful effects of aflatoxin B₁ on haematological parameters was achieved by adding mycosorbents to the compound feeds of broiler chickens (esterified glucomannan, clino-plotiolute) (Oguz et al., 2000; Aravind et al., 2003; Basmacioglu et al., 2005). The negative polarity of mycoxotins is attracted by the positive polarity of the toxin binder - a mechanism through which toxin are immobilized and eliminated from the organism of animals (Kana et al., 2014).

**Conclusion**

The supplementation of mulard ducks' feed with increasing doses of AFB, only 0.5 or 0.8 mg/kg feed) induced changes in haematological parameters with oligochromasia, erythropoiesis, reduced haematocrit, thrombocytopenia, leukocytosis with heterophilia and lymphocytopenia as well as was accompanied with bone marrow hypocellularity. The supplementation of the feed with 2 g/kg mycosorbent – Mycoxot NG to the feed containing 0.5 mg/kg AFB, reduced efficiently the harmful effect of AFB, on studied haematological indices and bone marrow lesions.

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Genetics and Breeding

Usability of metadata analysis of goat genetic resources among five countries from Africa, Asia and Europe: Metadata analysis of goat genetic

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The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods, Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

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