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Case report

PATHOLOGICAL CHANGES OF SELECTED ORGANS AND THE HEALTH CONDITION OF THE BROWN HARE (*LEPUS EUROPAEUS* PALL.) – A CASE REPORT

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Summary

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This paper describes a case of anatomopathological changes in almost all internal organs in a female brown hare shot in the western part of the Lublin Upland in Poland. The changes were observed in macroscopic examination and confirmed by necropsy. Bacteriological assays showed a high titre of *Enterobacter cloacae* and *Staphylococcus aureus* bacteria in the internal organs, and as a quite disturbing finding, *S. aureus* was detected as resistant to penicillin and susceptible to only one of the eight antibiotics used. Parasitological analysis showed the presence of three parasites in the intestines, one being a protozoan and two nematodes. The obtained data concerning only one case, therefore, do not reflect the health condition of the entire population, but only indicate potential threats to the epizootic condition in the population of this species. Taking into account the elements of the lack of veterinary tests of small game meat before introducing it for human consumption, the data on the described case indicate a potential risk to public health. Therefore, it would seem necessary to carefully assess any anatomopathological changes in hunted hares before making them available for consumption, and in case of any doubts, to subject the meat to such inspection.

Key words: Brown hare, pathological changes, Poland, public health

Over the past few decades, most European countries have seen a clear downward trend in the population of hares. No different is it in Poland, where on most hare hunting grounds, there is only a population of this species in near-catastrophically low numbers (Dziedzic *et al.,* 2002; Farkas *et al.,* 2016; Flis, 2016; Pavliska *et al.,* 2018; Sliwinski *et al.,* 2019).

Although it is difficult to determine the exact cause of this unfavourable trend, the most frequent mention is made of intensive changes in agrocenoses, which are the main habitat for this species. The in-

tensification of cropping, which impacts the heterogeneity of the hare habitat quite tangibly, combined with the growth in mechanisation of all farming operations and the widespread use of pesticides contributes to a significant impoverishment of the structure of the agrocenoses. Through these changes, multi-hectare monoculture plantations are becoming more common in the agricultural landscape, which are not optimal habitats for hares (Schmidt *et al.,* 2004; Smith *et al.,* 2004; Kamieniarz *et al.,* 2013; Hušek *et al.,* 2015; Panek, 2018).

Other factors that directly affect the population dynamics of this species significantly include predation. In this connection, many authors cite predation by free-living foxes as having the most influence, the number of which in many countries is consistently high (Dziedzic *et al.,* 2002; Panek *et al.,* 2006; Panek, 2009; Reynolds *et al.,* 2010; Demirbas, 2015). The high density of foxes in Poland is mainly due to a decrease in natural mortality resulting from the oral immunisation of this species against rabies carried out since 2002 (Flis et al., 2018). In addition, the negative impact of synanthropic predation does not fail to have a bearing on the number and, consequently, the functioning of the hare population (Nasiadka & Dziedzic, 2014; Flis & Rataj, 2019).

Numerous causes of disease both viral and bacterial, as well as numerous parasites, are also not without consequence for hare population dynamics. Among hare disorders, coccidiosis and other parasitic diseases are most often reported (Pikula *et al.,* 2004; Dubinsky *et al.,* 2010; Chroust *et al.,* 2012; Kornaś *et al.,* 2014; Nasiadka & Dziedzic, 2014). However, bacterial or viral diseases such as tularaemia, brucellosis, or EBHS (European brown hare syndrome) can also have considerable influence (Decors *et al.,* 2011; Flis *et al.,* 2016; Salvoli *et al.,* 2017). There are a number of other less common ones. In Italy in 2012 and Spain in 2014, a second type of rabbit haemorrhagic disease virus, RHDV2, was detected in isolated individual brown hares (Velarde *et al.,* 2017). This virus was also detected in France in 2013, where it is responsible for a third of all viral diseases in hares (Le Gall – Reculé *et al.,* 2017) and in Australia in 2016 (Hall *et al.,* 2017). In 2018, two cases of this virus were also detected in hares in England (Bell *et al.,* 2019) and one case in Central Scotland (Rocchi *et al.,* 2019). Studies of the health of the hare population in the western Lublin region revealed the occurrence of numerous anatomopathological changes affecting both sexes and different ages of animal. In addition, two strains of bacteria (*Nocardia* and *Providencia rustigianii*) were found in the internal organs, as well as in the testes. It should be emphasised that neither of the isolated strains of bacteria had been described in this species previously (Rataj *et al.,* 2019).

The material for analysis was a single brown hare shot on a hunt in the western Lublin region of Poland on November 3, 2019. It was an adult female weighing 5.1 kg, which did not show any morphological symptoms and had not shown any behavioural ones that signified a possible disease. The age of the hare was estimated by Stroh's sign, the absence of which qualified it as an adult (Stroh, 1931). The sex was determined by the appearance of the secondary sexual characteristics (Pielowski, 1979). During exenteration of the hare, numerous tumors were found on the surface of the internal organs, which prompted the hunter to submit the carcass for comprehensive veterinary examination (Fig. 1).

Fig. 1. Numerous abscesses on the surface of the liver.

The unusual appearance of the viscera, which included the liver, spleen, heart and lungs, was the reason for exploration to learn the cause of this problem. Material for histopathological investigation was taken from the organs listed above. This material was fixed in 4% buffered formalin, and then dehydrated and immersed in paraffin. Sections 4 μm thick were stained with haematoxylin and eosin (HE). The prepared slides were evaluated using an Olympus BX 43 light microscope, and photographic documentation of microscopic images was made using an Olympus SC microscope digital camera linked to a computer.

The stomach and small intestine were taken for parasitological analysis to confirm the presumed occurrence of parasites. The preparation of samples for bacteriological examination during the necropsy conducted in the field required the use of a transport kit, and for the purpose the BBL CultureSwab Plus system (Becton Dickinson France S.A.S.) was used with Amies transport medium. This kit consisted of a sterile bag with a tear-off closure containing a sample applicator, at the end of which was a rayon swab for taking the material, and also containing a test tube with transport medium, in which the sample applicator with the sample was placed.

Transport medium of this type which does not contain nutrients is buffered with phosphate, and the anaerobic environment is ensured by the presence of sodium thioglycolate. Complete protection against drying out was ensured by the moist transport medium (Table 1).

A bacteriological analysis was performed using various enrichment and differential media to identify individual species of microbes. The solid medium was agar with the addition of 5% sheep blood for the propagation of aerobic microbes and possible determination of haemolysis. Sugar broth was used as a liquid medium for propagating aerobic bacteria. Other media including Chapman, MacConkey and Sabouraud were also used to identify possible organ mycoses, and the study also used a medium with cetrimide to identify *Pseudomonas* microbes and a D-coccosel medium to identify faecal bacteria. Further identification of individual types of bacteria was carried out using bioMérieux API (Analytical Profile Index) biochemistry tests. Control of the capability of the media was carried out using sample strains.

Table 1. Composition of the Amies transport medium in 1000 mL of distilled water

Ingredient (chemical name)	Quantity (g)
Sodium chloride	3.0
Potassium chloride	0.2
Magnesium chloride	0.1
Calcium chloride	0.1
Di-sodium hydrogen phosphate	1.15
Monopotassium phosphate	0.2
Sodium thioglycolate	1.0
Bacteriological agar	7.5

An XA 110 analytical balance (Radwag) was used for accurate measurements of the mass of sections of the organs under study. The accuracy of this balance was guaranteed by automatic internal calibration that takes into account the passage of time and changes in temperature. To prepare representative samples, 10 mg of each tissue type was taken (liver, spleen, lungs, heart, and intestine). Saline in a 90 mL volume was added to each sample of a given organ and the whole was subjected to a homogenization process. Serial (geometric) dilution was performed of each of the five representative samples covering each tissue under study. Each of the dilutions of individual organs in an amount of 0.1 mL was inoculated onto two culture plates by the surface method and incubated.

When estimating colonies by counter, only those plates from subsequent dilutions that grew from 15 to 150 colonies were taken into account. A formula was used to determine the number of colonies which is commonly applied in this type of research (Libudzisz *et al.,* 2004 Malarska 2005):

$$L = \frac{C}{(N_1 + 0{,}1N_2) \times d} \times a$$

where: L – number of colonies in 0.1 mL (cfu/0.1 mL); C – sum of colonies on all plates selected for counting; N_1 – number of plates from the first dilution; N_2 – number of plates from the second dilution; d – dilution coefficient corresponding to the first lowest dilution; a – coefficient of the amount of material introduced onto the plate when inoculating 0.1 mL (a=10).

The susceptibility of the isolated bacterial strains to eight commonly used antibiotics was also analysed. The analysis was performed using the Kirby–Bauer disc diffusion method based on the diffusion of antibiotics in the discs into the medium, using the antibiotics in a concentration gradient. This method is used widely for testing drug resistance. A bacterium's susceptibility to a specific antibiotic is determined based on the size of the bacterial growth inhibition zone, it being defined as susceptible, intermediate, or resistant. The extent of susceptibility was determined from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) tables.

The following antibiotics and doses were used for the analyses: tylosin TY30 – 30 µg, amoxicillin/clavulanic acid AMC30 – 30 µg, gentamycin CN10 – 10 µg, penicillin GP10 – 10 µg, sulphamethoxazole/trimethoprim SXT25 – 25 µg, marbofloxacin MAR5 – 5 µg, oxytetracycline OT30 – 30 µg, and enrofloxacin ENR5 – 30 µg.

Study cases

The general macroscopic appearance of the internal organs selected for the study raised some questions. A detailed macroscopic examination of the liver revealed the presence of numerous lesions that covered almost all of its lobes. The forms of this type which were immediately visible were 2 mm to 5 mm in size and were located directly under its capsule (Fig. 1). On the other lobes, the location of the lesions was different and they only affected deeper sections of the liver, which was not visible without a preliminary incision of each of them. The location of these nodular forms, located inside the lobe, was at different depths from 1 to 3 cm. Several large lesions were cut open, and there was a semi-liquid or solid content of cream-pea-green colour. The number of liver lobes was normal and they had sharp edges and a dark red colour. One lobe was bi-coloured: it was mostly dark red with local pockets of yellowness, indicating

focal fatty degeneration. The consistency of this lobe in the places where it was yellow was clay-like.

A necropsy of the lungs revealed their structure to be normal. Individual lung lobes had rounded edges. One of the lobes did not have a uniform colour but was bicoloured dark and bright red. Striped petechiae were visible on one of the lobes, and the lung tissue had a friable consistency. The preliminary diagnosis based on macroscopic examinations of this lobe indicated emphysema.

A detailed macroscopic examination was also performed on the heart. The pericardial sac was found to contain a small amount of clear liquid coinciding with petechiae under the epicardium. The colour of the myocardium and the petechiae found under the epicardium provisionally indicated myocardial inflammation.

The spleen of the studied hare was of normal size, red in colour, and had sharp edges. The reticular structure of the spleen was preserved along its entire length, and the consistency of the studied organ was elastic.

To confirm the macroscopic changes, sections of the lungs, heart, liver, and spleen were taken for histopathological examination. Histopathological examination of the liver showed the presence of abundant purulent infiltration, consisting mainly of neutrophils (Fig. 2). Dark red and yellow-red liver sections showed signs of fatty degeneration and hyperaemia. Some hepatocytes in the middle region of the liver lobules showed signs of degeneration, progressing rapidly to liquefactive necrosis with cell cytolysis and nuclear pycnosis and karyorrhexis. In several areas affected by necrosis, the remains of decayed nuclei and cell membranes were visible.

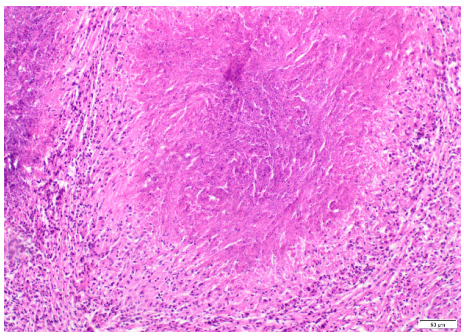

Fig. 2. Liver abscess. Haematoxylin and eosin (HE) staining, bar=50 μm.

Numerous purulent foci consisting of neutrophils were found in the lungs, indicating diffuse purulent pneumonia. In addition, the presence of parasites at different stages of development was observed in the alveoli and respiratory bronchioles. The presence of diffuse purulent infiltration consisting mainly of neutrophils and eosinophils was found in interstitial tissue, and numerous extravasations were also observed (Fig. 3). Hyperaemic foci were found in several places of the examined sections of lung tissue. Histopathological examination of the lung lobe with friable consistency showed ruptured alveolar walls, which indicated emphysema, and served only to confirm the macroscopic changes observed in the necropsy.

Inflammatory foci consisting mainly of mononuclear cells were found in the myocardium. The structure of the muscle fibres was unchanged and cell nuclei were clearly delineated (Fig. 4). There was significant hyperplasia of the fibrous connective tissue between the muscle fibres.

Histopathological examination of the spleen showed only a small degree of hyperaemia.

Bacteriological analysis of the collected material showed differences in the number of colonies of *Enterobacter clo-*

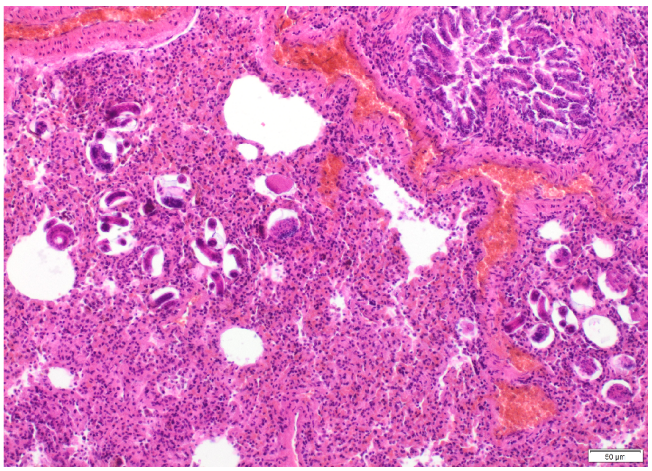

Fig. 3. Purulent pneumonia with parasites in the alveoli. Haematoxylin and eosin (HE) staining, bar=50 μm.

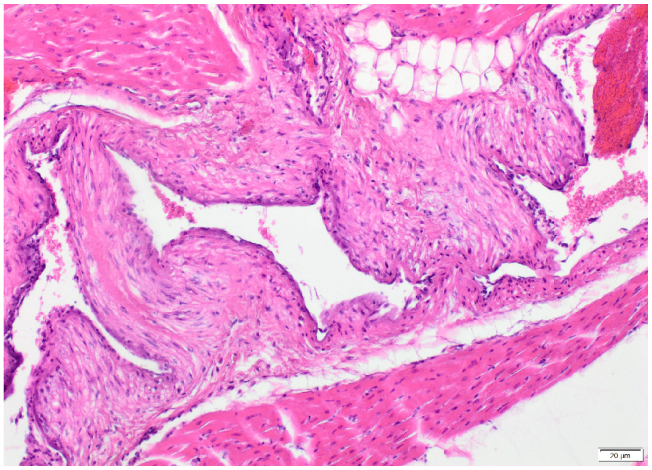

Fig. 4. Myocarditis with fibrous connective tissue spattering. Haematoxylin and eosin (HE) staining, bar=20 μm.

acae and *Staphylococcus aureus* bacteria in individual organs. With regard to the *Enterobacter cloacae* colonies found in the lungs, these were observed in numbers above the upper countable range in dilutions from 10^{-1} to 10^{-5} (Table 2). Colonies in numbers below the upper countable range were found at high dilutions, namely 10^{-6} and 10^{-7}. Such high dilutions allowed the number of colonies to be determined at 1.2×10^{8} cfu/mL. In the investigation of the myocardium, colonies in numbers above the upper countable range occurred in dilutions from 10^{-1} to 10^{-4}, and colonies below the range occurred in dilutions 10^{-5} and 10^{-6}. The results obtained in this way allowed the approximate number of colonies in the studied myocardium to be determined at 1.3×10^{7} cfu/mL.

A much lower number of *Enterobacter cloacae* colonies were found in the intestines and spleen. In the case of the intestines, colonies above the upper countable range occurred only in the two consecutive smallest dilutions, namely 10^{-1} and 10^{-2}. Inoculating material from two consecutive dilutions, i.e. 10^{-3} and 10^{-4}, the calculated number of colonies allowed the number of colonies in the myocardium to be determined at 9.2×10^4 cfu/mL. At dilutions from 10^{-5} to 10^{-7}, fewer than 15 colonies were seen or none were at all. The fewest colonies of *Enterobacter cloacae* were found in the spleen. Colonies in numbers below the upper countable range in two consecutive dilutions could be observed at the lowest dilutions of 10^{-1} and 10^{-2}. In subsequent dilutions from 10^{-3} to 10^{-7}, there were only individual colonies or there were none. The total number of colonies in the spleen was the smallest of all colony numbers in the studied organs and amounted to 9.1×10^2 cfu/mL. In subsequent dilutions inoculated liver material showed a complete absence of *Enterobacter cloacae* bacteria.

Bacteriological studies to determine the number of *Staphylococcus aureus* colonies produced similar results. Fewer than 15 colonies or their complete absence was observed in all dilutions on all culture plates with liver sample inoculations, which was similar to the result obtained in the investigation of *Enterobacter cloacae.* The presence of *Staphylococcus aureus* colonies in the intestine was not noted in any of the subsequent dilutions either. In the lungs, colonies of this bacterium numbering above the upper countable range

Table 2. Number of colonies of *Enterobacter cloacae* and *Staphylococcus aureus* bacteria in individual organs

Type of bacteria	Number of bacterial colonies on two plates in separate dilutions							Cfu/mL
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Lungs								
Enterobacter cloacae	x	x	x	x	x	246	31	1.2×10^8
Staphylococcus aureus	x	x	x	x	x	211	z	9.6×10^7
Heart								
Enterobacter cloacae	x	x	x	x	260	30	z	1.3×10^7
Staphylococcus aureus	x	x	x	x	190	31	z	1×10^7
Intestine								
Enterobacter cloacae	x	x	171	33	z	z	z	1.1×10^4
Staphylococcus aureus	–	–	–	–	–	–	–	–
Spleen								
Enterobacter cloacae	170	32	z	z	z	z	z	9.1×10^2
Staphylococcus aureus	114	z	z	z	z	z	z	5.2×10^2
Liver								
Enterobacter cloacae	–	–	–	–	–	–	–	–
Staphylococcus aureus	–	–	–	–	–	–	–	–

x – colonies above the upper countable range; z – fewer than 15 colonies or no colonies.

Table 3. Susceptibility of isolated bacteria to antibiotics

Antibiotic used	Bacterium	
	Staphylococcus aureus	*Enterobacter cloacae*
Tylosin TY30	IR	R
Amoxicillin/clavulanic acid AMC30	S	R
Gentamicin CN10	IR	R
Penicillin GP10	R	R
Sulphamethoxazole/trimethoprim SXT25	S	S
Marbofloxacin MAR5	IR	S
Oxytetracycline OT30	IR	IR
Enrofloxacin ENR5	IR	IR

S – susceptible; IR – intermediately resistant; R – resistant

were found in dilutions from 10^{-1} to 10^{-5}. Colonies in a density below the upper countable range and suitable for further calculations were only present in the dilution $10^{-6.}$ The total number of bacterial colonies here was 9.6 x 10^{7} cfu/mL. Analysis of the material in the form of myocardial tissue in dilutions from 10^{-1} to 10^{-4} showed the presence of colonies above the upper range, while colonies below the range were evident in the next two consecutive dilutions, i.e. 10^{-5} and 10^{-6}. In the next dilution in the series, that is, 10^{-7}, the presence of colonies of this bacterium was not noted. The number of bacterial colonies in the myocardium was 1×10^{7} cfu/mL. By far the lowest number of colonies occurred in the spleen. Colonies were only evident in the first and lowest dilution, that is, 10^{-1}, and numbered below the range. In subsequent dilutions of material taken from the spleen, fewer than 15 colonies or a complete absence of them were found on the culture plates which were inoculated. Plates with inoculated material that were included in the calculations indicated the presence of bacterial colonies in the spleen in an amount of 5.2×10^{2} cfu/mL.

The study also included assessment of the susceptibility of the isolated bacterial strains to antibiotics in vitro (Table 3). An isolated strain of *Staphylococcus aureus* was found to be resistant to one of the antibiotics used, intermediately resistant to five, and sensitive to amoxicillin/clavulanic acid and sulphamethoxazole/trimethoprim. The *Enterobacter cloacae* strain was resistant to four antibiotics and susceptible to sulphamethoxazole/trimethoprim and marbofloxacin.

Parasitological studies of the gastrointestinal tract, including the stomach and small intestine, large intestine, and caecum, showed the presence of parasites at different stages of development. Only the stomach was not found to contain any parasites. The presence of *Eimeria sp.* oocysts, individual eggs of *Trichostrongylus sp.,* and mature forms of the latter parasite were observed in the small intestine. Individual larvae of the parasite *Passalurus sp.* were also confirmed. Parasitological examination of the large intestine revealed the presence of *Eimeria sp. oocysts* and individual eggs of *Trichostrongylus sp.*, similar to the findings in the small intestine. The presence of *Passalurus sp.* larvae was observed in much larger numbers than in the small intestine. No mature forms of *Trichostrongylus sp.* were found in the large intestine, the oc-

currence of which was previously detected in the small intestine. A parasitological investigation of the caecum revealed the presence of *Eimeria sp.* oocysts., individual eggs of *Trichostrongylus sp.*, and a small number of *Passalurus sp. larvae.*

The thorough, detailed parasitological study made it possible to discern an additional four foreign bodies (buckshot) in a segment of the small intestine. It should be noted that the location of bullets and buckshot is a rather problematic task in some cases and is not feasible without a preliminary X-ray image. Finding buckshot was an entirely secondary matter, although not incongruent, and it was consistent with previous information about the use of a firearm to kill the animal in question. In this case, buckshot was found in various locations in the small intestine, where the light microscope revealed one ball in its middle segment. The location of the three remaining balls touched only the outer part of the intestine including the mucosa. Extravasation and segmental hyperaemia of a short segment of the small intestine 3 cm long were observed in this layer.

The high titre of the colonies of the detected bacteria *Enterobacter cloacae* and *Staphylococcus aureus* in the internal organs indicates the possibility of the risk of an epidemic being raised by the consumption of untested hare meat, which is usual practice for hunters. The presence of *Staphylococcus aureus* in the heart and lungs, which are edible organs, is very disturbing. The bacteria were resistant to penicillin and susceptible to only one of the eight antibiotics used. In addition, it is not without significance that staphylococci produce enterotoxin resistant to high temperatures, and, consequently, even to cooking or roasting when in infected meat, and therefore the possibility of transmission of bacteria to humans is not precluded. In addition, the isolated strain of *Enterobacter cloacae* was resistant to four and susceptible to only two of the eight antibiotics applied.

The parasitological analysis showing the presence of three parasites in the intestines, one a protozoan and two nematodes, also indicates the potential for infection by these parasites. Even granting that the parasites of the group *Passalurus sp.* are specific only to hares and do not pose a threat to public health, the two other parasites can occur in humans.

At the same time, it should be stated that the presence of bacteria in the internal organs and parasites *Eimeria sp., Trichostrongylus sp., Pasalurus sp*. In the intestine of the described hare confirms the occurrence of numerous diseases with a diverse etiological background in this species, which have a significant impact on the dynamics of the population size. Moreover, due to the fact that small game meat usually does not undergo official veterinary inspection before being placed on the market, cases such as the one described, although they pose a risk to public health, should be treated as special and at the same time exceptional. They should not be generalized as the health status of the entire population. Nevertheless, any noticed anatomical and pathological changes should not be underestimated, as they may indicate the occurrence of a disease state and thus pose a threat to public health.

Thus, it is necessary to carefully assess the internal organs of any animal before consuming it, and in case of any doubts, submit it to a thorough veterinary-sanitary inspection or condemn it as unfit for human consumption.

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