NEW DEVELOPMENTS IN VETERINARY MEDICINE

Editor

Prof. Dr. Tünay KONTAŞ AŞKAR

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Veterinary Medicine

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PREFACE

Veterinary medicine is a branch of medicine related to animal health, public health, preventive medicine, food safety. Most human infectious diseases (about 60%) are caused by animals. These diseases are called as zoonosis (such as COVID-19, Ebola, HIV, SARS, MERS, Avian flu) and they can be transmitted from animal to human or from human to animal. Therefore veterinarians are very important in protection of public health. Veterinarians also take significant role in ensuring food safety from farm to fork. And the diseases of animals can be treated by only veterinarians.

Nowadays, innovations in technology and science occurs in the field of health sciences also. While health awareness increases in society with modern life, the incidence of diseases such as obesity, diabetes and cardiovascular diseases increases due to lack of movement, increased consumption of ready-made and additive foods. For this reason, veterinary medicine, which has important responsibilities in the protection of public health, needs to update itself by following technological and scientific developments. In this book; it is aimed to present recent developments in veterinary medicine.

Hoping to be useful to readers with this book.

Prof. Dr. Tünay KONTAŞ AŞKAR
Editor

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CHAPTER I

APOPTOTIC EFFECT OF MEDLAR ON DIABETIC RATS

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1. Introduction

Diabetes mellitus (diabetes) is a chronic disease characterized by hyperglycemia depending on carbohydrate, protein, and lipid metabolism disorders (1). Disorders of glucose metabolism (e.g. hyperglycemia) leads to excessive free radical production and oxidative stress under stress conditions. Oxidative stress causes cell loss by leading to a process called apoptosis, which is defined as programmed cell death of various cell types and necrotic cell death (2). Apoptosis is a form of genetically programmed cell death in organism cells which have nuclei. It is very important in extinction phases of cells which become harmful to the organism (3).

Scientists are investigating the efficiency of plants for the treatment of the diabetes and Turkey has very rich plant diversity. There are many fruit varieties in Turkey and one of these fruits is Medlar (*Mespilus germanica*). This fruit has origins from North Anatolia, Southeast Europe, and Iran, and its tree has a bush shape with an average 2-6 meters height. Medlar is belongs to the Rosaceae family (4). In traditional medicine, this plant is often used to treat microbial infections, wounds and diabetes. Also, Medlar's fruits are rich in phytochemicals, nutrients and therapeutic substances (5). But there is limited data about the efficiency of Medlar on apoptosis mechanism of diabetes. Therefore the aim of this study is to determine the effect of Medlar leaf extract on apoptosis mechanism of diabetic rats.

2. Material and Methods

For the study, medlar leaves were collected and dried in room temperature. Then leaves put in an oven at 40 °C for 18 h. Then dried plant material was powdered by using a mechanical blender. Then ethanol (70%) extraction was performed on the leaf powder with a Soxhlet apparatus. After filtration of the extract with Whatman filter paper, the filtrate was placed in a rotary evaporator for evaporation. Obtained extract stored at 4 °C for study.

In this study, 28 male Sprague-Dawley rats between 21-22 weeks old and 260-350 g weight have been used as material. The rats fed with tap water and standart pellet and ad libitum in the rooms. Rats were left for adaptation with tap water and standard rat feed for 7 days. At the end of this period, initial fasting blood sugar values of rats were measured by a glucometer (One Touch Lifescan, America).

Then rats were divided into 4 groups: Control, Medlar, Diabetic, and Diabetic+Medlar group. Control group was fed with standart feed and water for 4-weeks trial. 100 mg/kg Medlar extract per rat was added to drinking water of Medlar groups for 4 weeks. In order to induce diabetes in diabetic groups, a single dose of 65 mg/kg streptozotocine (STZ) was applied to each rat intraperitoneally (*i.p.)* for the formation of diabet. Two days after the injection, rats having 250 mg/dL or above measured blood glucose level were considered as diabetics.

For the determination of changes in serum glucose and caspases (3 and 8) levels in groups at the end of 4-week study period, blood samples were taken from the heart of rats. Then samples were centrifuged in 2500 rpm for 10 min and serums were obtained. Obtained serum samples were stored at – 80 ^{0}C.

Rat-specific ELISA kits based on biotin dual antibody sandwich technology were used for the determination of Caspase 3 (Cat No. YHB0311Ra, YH Biosearch Laboratory, China) and Caspase 8 (Cat No. YHB124 Ra, YH Biosearch Laboratory, China) activities in serum samples. The results were calculated as ng/L. "SPSS 17.0" package software was used for statistical analysis of data. Statistical differences among groups were evaluated using the Student *t*-test. The results obtained were given as X (mean)± S_e(Standard error). P<0.05 and below values are accepted as statistically significant.

3. RESULTS

In this study we aimed to investigate the effect of Medlar on glucose metabolism, and apoptotic markers in diabetic rats. And changes in blood glucose and caspases (3 and 8), levels of the rats in experimental groups (control, Medlar, diabetic, diabetic+Medlar) were given in Table 1.

Table 1. Changes Glucose Metabolism and Apoptotic Parameters in Rat Experimental Groups

Parameters	**Control Group n=7**	***Medlar* Group n=7**	**Diabetic Group n=7**	**Diabetic+ Medlar Group n=7**
Blood glucose (mg/dL)	77.6±8.5[a]	80.4±6.2[a]	302.5 ±35.2[b]	238.3±31.5[c]
Caspase-3 (ng/L)	5.36±1.02[a]	6.12±1.36[a]	11.54±2.8[b]	8.05±1.04[c]
Caspase-8 (ng/L)	12.26±3. 25[a]	11.74±4.38[a]	27.01±6.35[b]	18.77±6.49[c]

[a, b, c]: Groups with different letters on the same line are important.

In present study, we determined the changes in the levels of blood glucose. While blood glucose levels were relatively high in the diabetic group when compared with the control group, these levels were found lower (p<0,01) in diabetic+Medlar group when compared with diabetic group. For the determination of apoptotic changes in the study, we measured the activities of caspase 3 and caspase 8 enzyme activities. However caspase-3 and caspase-8 levels of diabetic group were found significantly higher (p<0.001) than the control group, in diabetic+Medlar group caspase-3 and caspase-8 levels were found significantly lower (p<0.001) when compared with the diabetic group (Figure 1).

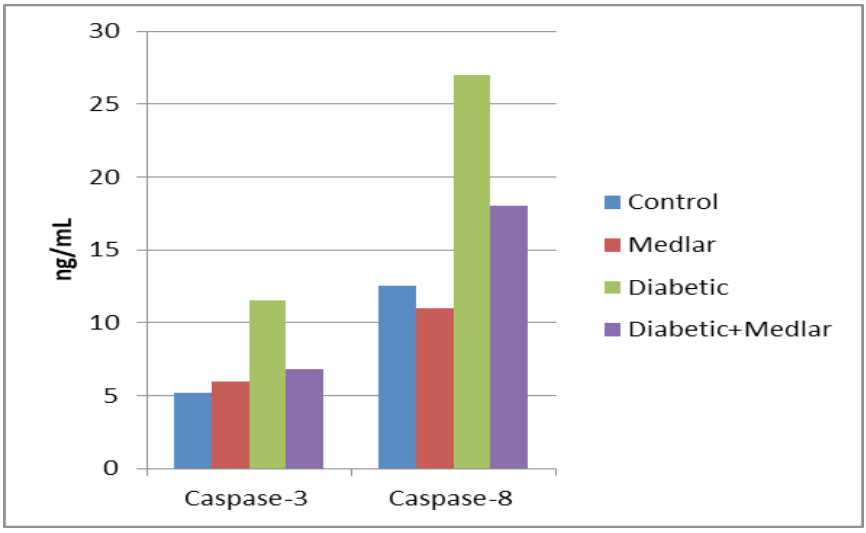

Figure 1. Changes in Caspase-3 (ng/L) and Caspase-8 (ng/L) Levels in Rat Experimental Groups

4. Discussion

In this study, serum glucose, caspase-3, and caspase-8 levels were found significantly lower in diabetic+Medlar group when compared with the diabetic group. When cells become older or when these cells turn into a threat for the organism, apoptosis process which is defined as the programmed cell death, is activated. Apoptosis begins with the activation of caspase enzymes, members of protease family (6).

Caspases mediate apoptosis in Type 2 DM (7). And it has been determined in some studies that high glucose levels affect various phases in the apoptotic signal. Also it has been demonstrated that increased caspase 3 and 8 activities in patients with Type 2 DM can be inhibited by an antidiabetic agent, namely metformin (8). But increase in caspase enzymes cause cell death. In the present study, Medlar supplementation were lowered the caspase 3 and caspase 8 activities, which were higher in diabetic rats. This situation may be related with the regression of apoptotic process by Medlar supplementation in diabetic rats.

5. Conclusion

There are many studies about the plants effects on insulin resistance and diabetes, but there is limited study about the effect of *Medlar* leaf/fruit exctract on

diabetes. In this study, Medlar supplementation reduced blood glucose levels and regression in the apoptotic process occured by an increase in insülin stimulation from pancreatic cells and elevation in insülin effectiveness at receptor levels in the targeted cells. With the light of the data obtained from the study, it is considered that Medlar may be used in the treatment of insulin resistance and diabetes in the following years, but there is need for further studies.

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CHAPTER II

LATERAL FLOW IMMUNOASSAY: CREATIVE DIAGNOSTICS DISCOVERY FOR VETERINARY MEDICINE

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1. Introduction

Depending on the developing technology and the increase in the human population, the needs of the societies also differ. This situation also increases the demand for diagnostic tests where people can get quick results. To date, liquid chromatography-mass spectrometry (LC/MS), gas chromatography-mass spectrometry (GC/MS), high-pressure liquid chromatography (HPLC), real-time polymerase chain reaction (qPCR), Enzyme linked immunosorbent assay (ELISA), and biosensors have been developed to meet the needs (1, 2). Scientific research has focused on the development and optimization of portable, easy-to-use, cost-effective and user-friendly methods for point-of-need (PON) testing with rapid results (3-5).

The lateral flow method is commonly known as the immunochromatographic method because it uses immunological components (antibody/antigen) that move across the membrane surface via capillary action (6). The method is based on the reaction of an analyte/antigen (Ag) with a selective antibody (Ab) to form an Ab-Ag complex. The effectiveness of the test depends on the efficiency

of Ab-Ag complex formation and its ability to detect the rate of formation. (7). Lateral flow immunoassay (LFA) are paper-based immunochromatographic card tests that can be used to detect and to quantify the presence of analytes in mixtures where the sample is placed in a test kit and results are displayed for an average of 5 to 30 minutes. LFA-based tests are used for qualitative and quantitative analyzes of specific antigens, antibodies and gene amplification products in a variety of biological samples such as serum, plasma, whole blood, urine, saliva, sweat and other fluids (8).

The technical basis of the LFA was first developed by Plotz and Singer in 1956 as a latex agglutination test (9). In 1959, Yalow and Berson determined insulin in human blood plasma using parafin paper with the diagnostic principle based on the lateral flow test (paper chromatography) (10). The human pregnancy test was developed in the 1970s thanks to the understanding of the biology of human chorionic gonadotropin (hCG) in studies by Vaitukaitis et al. (11). In the late 1980s, the first lateral flow tests and necessary materials were introduced to the market. From those years, technology applications and industry continued to evolve. With the year 2006, a large market area has been created around the world thanks to developing technology and industry applications. The application of the technology has expanded into various fields such as veterinary medicine, agriculture, food, biological warfare, molecular diagnostics and theranostics, industrial testing, environmental health and safety, along with clinical diagnostics (12).

2. Principle of the Lateral Flow Immunoassay

LFA tests are one of the simplest and most widely used biosensors for analytical detection. The method is based on technologies which rely on a visible color or instrument detected change to identify the presence or absence of specific molecules. The principle of LFA is simple: a liquid sample containing the analyte of interest operates on the basis of liquid movement through various polymeric strip regions to which molecules are added that can interact with the analyte without the aid of external forces (capillary action). LFA strips contain specific sections with antibodies to which the analyte will or will not bind, depending on the desired biomarker (13). As a paper-based chromatography, LFA requires a sample pad, a conjugate pad, a nitrocellulose membrane, an absorbent pad (Figure 1).

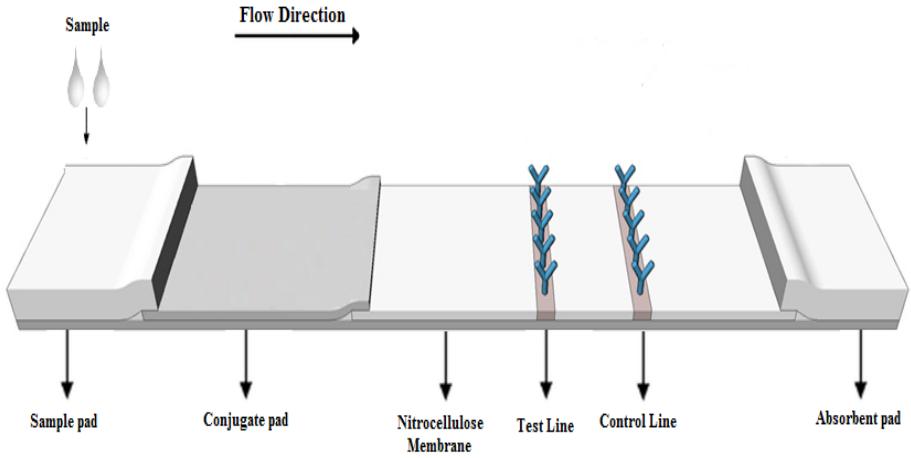

Figure 1. Typical view of LFA test strip

Generally, a typical LFA has a sample pad just below the sample drop area. The dripped sample first accumulates in this pad. Since the sample is dripped in excess of the liquid holding capacity of this pad, the liquid moves towards the conjugate pad that contacts this pad. The conjugate pad is covered and the colored molecules are contained in this pad. The liquid sample coming to the conjugate pad reacts with the molecules there, dragging the molecules in this pad and moving towards the nitrocellulose membrane. The first portion of the nitrocellulose membrane is covered, but the portion containing the subsequent test and control line is uncovered; The test result can be observed here. The liquid sample coming to the nitrocellulose membrane also drags the molecules in it and moves towards the end of the membrane based on the capillary current force on the membrane. Here, the relevant reaction stake place on the test line and control line, and the colored lines indicating the test result are formed. The liquid progresses further and accumulates in the absorbent pad, which contacts the end of the membrane, and this area is also covered (14, 15). To maintain the integrity of the pads and nitrocellulose membrane, a plastic base is placed at the bottom as a backing board and all parts are fixed on this plastic (Figure 2.a.). Results are read visibly or using a reader (Figure 2.b.). The presence or absence of captured conjugate lines is interpreted through there action matrix (12).

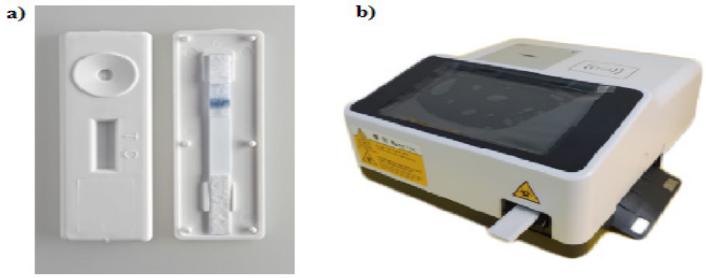

Figure 2. a) General view of the cassette **b)** The fluorescence analysis device.

3. Lateral Flow Assay Components

3.1. The Sample Pad

The main function of the sample pad is to provide an even and controlled distribution of the sample and to guide the sample to the conjugate pad. It is possible to reduce non-specific binding, to increase sample viscosity or to change pH by using components such as proteins, detergents, buffer salts, surfactants in order to control the sample flow rate. The sample pad typically consists of a cellulose fiber or woven mesh. Woven webs have a low bed volume while cellulose fiber pads have a much larger bed volume. In addition, the tensile strength of woven nets is higher than that of cellulose fiber (8, 12).

3.2. The Conjugate Pad

The conjugate pad is a pad made of fiber glass, polyester, or rayon material on which the detection reagent is held stable. After the sample pad is saturated, the sample flows towards the conjugate pad, where it releases the detection reagent; the detection reagent then leaves the conjugate pad and moves with the sample to wards the nitrocellulose membrane. The main role of the conjugate pad is to ensure the transfer of the sample and detection reagent, and to protect the conjugate after drying and rewetting (8, 12, 16).

3.3. The Membrane

The membrane is considered the most important element in LFA strips, and the most commonly used material is nitrocellulose. Polyvinylidene fluoride (PVDF), polyethersulfone (PES) cellulose acetate and charge modified nylon can also be used as membranes. The pore size of nitrocellulose membranes ranges from 0.05 to 12 μm and a number of nitrocellulose are available. Membrane selection is important for LFA. Because the pores are not evenly distributed (due to the

manufacturing process), the capillary flow time (typically seconds/cm) is a more accurate parameter. Capillary flow time can affect the sensitivity, specificity and consistency of the test line of the LFA test. The main purpose of the membrane in LFA is to maintain their stability and activity by binding the proteins in the test and to control areas throughout the shelf life of the product. The membrane should accept the conjugate and sample from the conjugate pad and drain it into there action area, allow there action to occur in the test and control lines, and allow excess liquids, label, and reactants to reach the absorbent pad (17, 18).

3.4. The Wicking Pad/ Absorbent Pad

It is a pad made from cellulose filters and found at the end of the LFA test. The main function of this pad is to prevent back flow of the sample enteringthe test strip (8).

3.5. The Plastic Cassette

In LFA, a plastic base is placed at the bottom to maintain the integrity of the pads and nitrocellulose membrane, and all other parts are fixed onto this plastic cassette (8).

3.6. Antibody

One of the most important factors in LFA is the selection of appropriate antibodies. The specificity of the antigen to bind to the antibody should be defined by the variable region of the antibody. The antibody can bind to the same epitope on multiple targets and perform non-specific bindings, resulting in misinterpretation of results. It is there fore important to select an antibody that recognizes a single target. For this, monoclonal antibodies are more preferred (19-21).

3.7. Labels

The most commonly used detection particles in LFA are gold nanoparticles. Apart from this, latex beads (mono disperse latex), fluorescent labels, enzymes, other colloidal metals and magnetic particles can also be used. It is essential that these particles be of uniform size and have a regular spherical shape when working with gold nanoparticles or latex beads. Because of the smaller size of the gold particles (20-40 nm), they show greater sensitivity than colored latex particles (100-300 μm). As a result, higher packing density can be achieved in a test line. In reader-based LFA, higher sensitivity can be achieve dusing fluorescent or paramagnetic particles (17, 22-24).

4. The Assay Formats

There are two different formats used in LFA. The first is the direct (sandwich) assay format and the second is the competitive assay. Generally, formats involve the formation of a complex between a detection reagent that is free in the LFA sample stream and a capture reagent that binds to the membrane in the test line (21).

4.1. Sandwich Format

In the sandwich format, the analyte must have more than one antigenic determinant region. Human serum albumin is more suitable for the detection of large molecules of protein nature, such as antibodies, which have more than two epitopes or binding sites. In this format, the conjugate pad contains a monoclonal or polyclonal antibody specific to the analyte, which is colored by binding the colored molecule. These antibodies are not stable on there action pad and these colored antibodies are dragged towards the test line with the in coming liquid sample. Another type of antibody specific to the analyte is fixed in the test line area (Figure 3.). In sandwich format, if a double line is seen in the observation window, the result is positive; if a single line is seen, the result is negative (Figure 5a). Larger analytes with multiple antigenic sites such as hCG, Dengue antigen or human immunodeficiency virus (HIV) are produced according to the sandwich model (25-30).

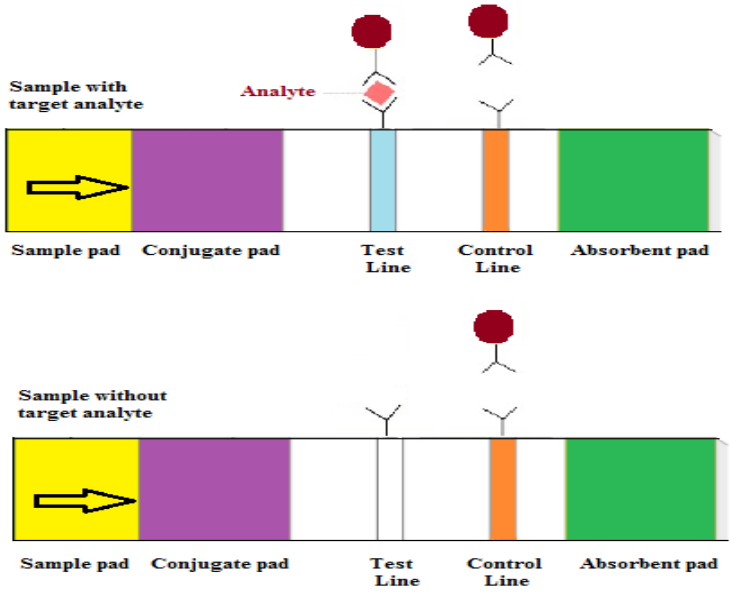

Figure 3. Sandwich format of LFA

4.2. Competitive Format

The competitive format is used in the analysis of analytes with only one antibody-producing region (antigenic determinant region) and in the analysis of low molecular weight compounds that cannot bind to two antibodies simultaneously. In this model, there is an unstable monoclonal antibody specific to the analyte, which is colored by binding the colored molecule on the conjugate pad. These antibodies are unstable and drift towards the test line with the in coming liquid sample. In the test line area, the analyte that will be analyzed is fixed. In the competitive model, if a single line is seen in the observation window, the result is positive; If a double line is seen, the result is negative (Figure 5b.) (25, 26, 30, 31).

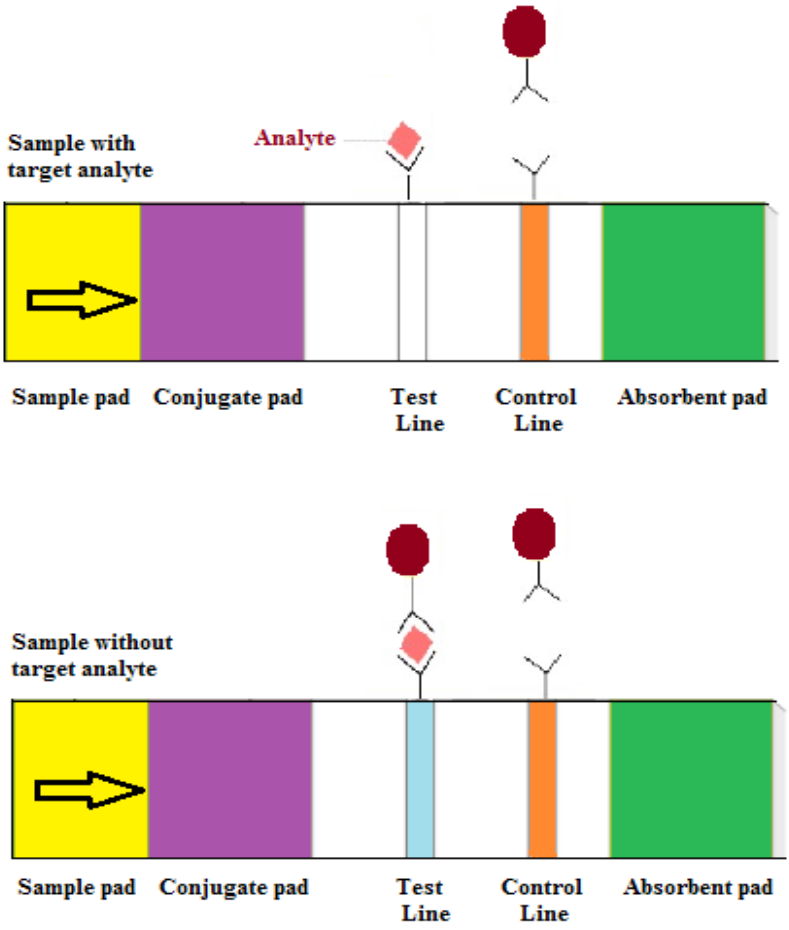

Figure 4. The competitive format of LFA

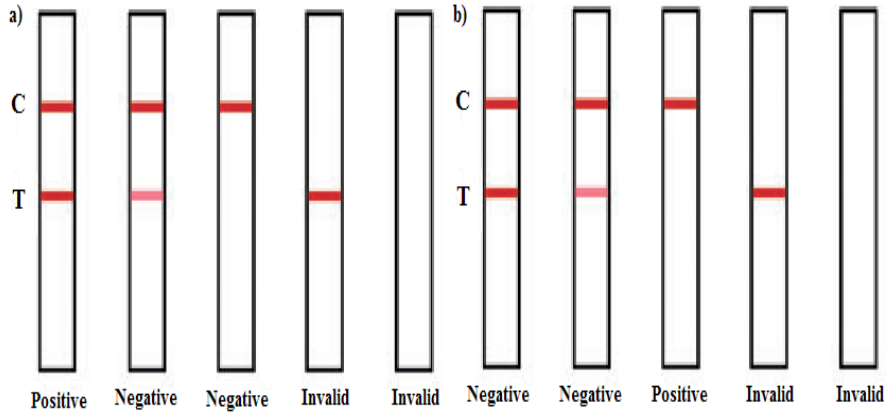

Figure 5. a) Labeled lateral flow strip of sandwich format in LFA **b)** Labeled lateral flow strip of competitive format in LFA

5. Application Areas of the LFA Method in Veterinary Medicine

Due to the high sensitivity, safety and ease of use of LFA test kits, they are critical in identifying dangerous infections, diseases and outbreaks in veterinary and clinical applications.

Wang et al. developed a competitive format LFA test strip for the detection of sulfonamides in egg and chicken muscle. Susceptibility to sulfonamides (sulfamonomethoxin, sulfamethoxydiazine, sulfadimethoxine, and sulfadiazine) was determined through an optical density scanner (at least 10 ng/mL). This method determined the differences between paralel analysis of test strips and HPLC in 27 egg samples and 28 chicken muscle samples for the detection of sulfonamides. As a result, it was stated that the test is fast and accurate for the detection of sulfonamides in egg and chicken muscles. It has been reported that a series of test strips are likely to be developed in the future for the detection of sulfonamides in food products of animal origin (32).

Brucellosis is an important zoonosis that is caused by brucella group microorganisms in farm animals such as cattle, sheep, goats, pigs and rams, which has negative effects on animal production in many countries of the world and threatens human and animal health. A study on cattle aimed to develop an LFA that will beused in the serological diagnosis of bovine brucellosis. The sensitivity, specificity, and diagnostic accuracy of LFA were evaluated in comparison with Rose Bengal Plate Test (RBPT), Complement Fixation Test

(CFT), and i-ELISA. Of 91 serum samples, 37 positive and 54 negative, and it was detected that 44 (48.3%) by i-ELISA, 34 (37.4%) by LFT, 45 (49.5%) by RBPT and 37 (40.7%) by KFT were positive. The complement fixation test was accepted as the reference test, and the sensitivity and specificity of LFA were determined as 92% and it was concluded that the developed test is a specific test that can be easily applied in the field and provides rapid results (33).

In human brucellosis, antibodies specific to immunoglobulin M (IgM) and immunoglobulin G (IgG) develop. In the test developed for human brucellosis, it was aimed to detect Brucella-specific IgM and IgG antibodies. By examining the diagnostic values of these tests, it was reported that the tests detect acute, persistent and recurrent disease. Sensitivity and specificity of Brucella IgM and IgG tests were determined as 96% and 99% (34).

In a Portuguese study by Abdoel et al., four simple and rapid LFA tests were developed for the serodiagnosis of brucellosis in cattle, goats, sheep and pigs. In the study, samples were taken from 37 cows, 48 goats, 68 sheep and 33 pigs. The sensitivity of lateral flow experiments based on the results obtained was determined as 90%, 100%, 90% and 73%, respectively. No reaction was observed in samples taken from non-brucello herds. It was stated that the easy use of these developed tests will provide convenience for the use of migrant farmers or in remote areas where Access to laboratory facilities is problematic (35).

Cryptococcosis is a type of fungal infection in humans and animals. It was studied in cats, dogs and koalas suspected of having *cryptococcosis* through the LFA test used in the diagnosis of *cryptococcosis* and developed for human patients. 528 serum samples (129 cats, 108 dogs, 291 koalas) were collected and evaluated by LFA. The sensitivity of LFA was 92%, 100% and 98% in cats, dogs and koalas, respectively. The specificity of LFA was determined as 81%, 84% and 62%, respectively. As a result, it was stated by the researchers that the most appropriate role for LFA could be a screening test to rule out the diagnosis of *cryptococcosis* in cats, dogs and koalas (36). Another study for the diagnosis of *cryptococcosis* in dogs and cats evaluated the performance of the 2-point-of-care (POC) *cryptococcal* antigen test on serum collected from dogs and cats. Compared with the *Cryptococcal* antigen latex agglutination system (CALAS). 102 serum samples were taken from 51 dogs and 40 cats. When the results of LFA tests and CALAS were compared, the sensitivity and specificity of theCrAg LFA POC test were 92% and 93% respectively, and the sensitivity and specificity of the CryptoPS POC test were 80% and 95% respectively (37).

The Active Anthrax Detect Rapid Test lateral flow immunoassay is a point-of-care test used to detect *Bacillus anthracis* capsular polypeptide in human blood, serum and plasma. The study evaluated the animal carcasses from the hippopotamus (*Hippopotamus amphibius*) and Cape buffalo (*Syncerus caffer*) during an anthrax epidemic in Namibia. The test showed a specificity of 82% and a sensitivity of 98% (38).

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite that is globally distributed and causes *Toxoplasmosis* by infecting humans and a wide variety of warm-blooded animals (39,40). 318 blood samples were taken from stray dogs and cats, and at the end of the study, a loop-mediated isothermal amplification- Lateral-Flow-Dipstick (LAMP-LFD) Device was developed for *T. gondii* infection. The current infection prevalence of *T. gondii* detected by LAMP-LFD device was 4.76% and 4.69% in stray cats and dogs, respectively (41).

Canine coronavirus is a single-stranded RNA virus that causes mild to severe enteritis in dogs of allages (42,43). In the study, out of 179 stool samples collected from dogs, 58 were positive for canine corona virus and 121 were negative. The sensitivity and specificity of the rapid test kit on the samples in he study was determined as 93.1% and 97.5%, respectively, to determine the accuracy of the kit (44).

6. Conclusion

By showing sensitivity and specificity in clinical, food safety, veterinary and environmental tests, LFAs have become one of the point of need tests that can provide fast and easy results without the need for laboratory equipment. Continual improvements in the sensitivity and reproducibility of the test are ongoing. The most important thing is that these tests can be used effectively outside the laboratories. It will continue to provide ease of use in field studies of veterinary practices and in traditional clinical settings.

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CHAPTER III

MELATONIN: THE ROLE IN SMALL RUMINANT REPRODUCTION

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1. Introduction

Melatonin was first discovered in 1958 by Aron B. Lerner from the pineal gland (1). Observing that bovine pineal gland extracts cause the melanophores in frog skin to appear white, Lerner named this substance "melatonin" by combining the Greek words "melas" meaning black and "tosos" meaning work (2, 3).

Melatonin synthesis begins with the uptake of tryptophan from the circulatory system by pinealocytes. Tryptophan in pinealocytes is first converted to 5-hydroxytryptamine (Serotonin) as a result of various enzymatic reactions. Serotonin is converted to N-acetylserotonin by N-Acetyl Transferase (NAT) and finally to melatonin by the enzyme Hydroxindole-O-Methyl Transferase (HIOMT). The step that is catalyzed with NAT is the speed limiter. Expression of the genes encoding these enzymes is weak during the day and strong during the night. Therefore, the epiphysis's melatonin secretion displays a circadian rhythm (4-6).

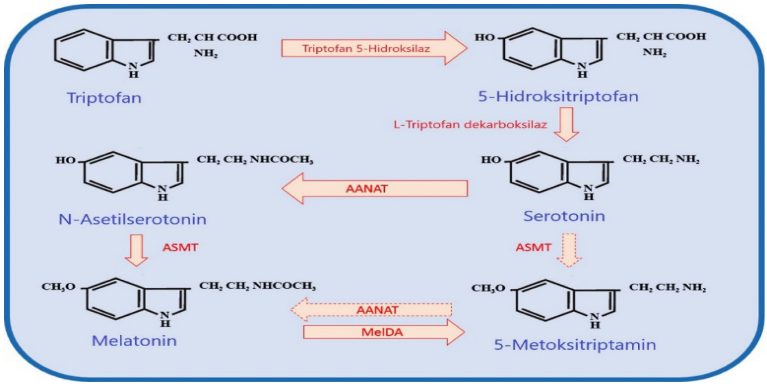

Figure 1. Classical biosynthesis pathways of melatonin in vertebrates (7).

1.1. Secretion, Functions and Chemical Structure

Melatonin is synthesized and secreted in cells and tissues such as the gastrointestinal tract, bile, skin, retina, spleen, testis, salivary glands, platelets and lymphocytes, most of which are from the pineal gland between the cerebral hemispheres in the mammalian brain (8). It plays a role in many physiological events depending on the tissue it is synthesized, such as regulation of the retinal response to the day-night variation in photoreceptors in the retina, the change of pigment granules in the skin and the protection of deep tissues against the harmful radiation of the sun, the protection of the integrity of the gastrointestinal tract with antiulcerative effect in the gastrointestinal tract and the oxidative effects of the bile ducts against oxidized cholesterol derivatives and bile acid in the bile. The most important function in small ruminants is the regulation of reproduction (2).

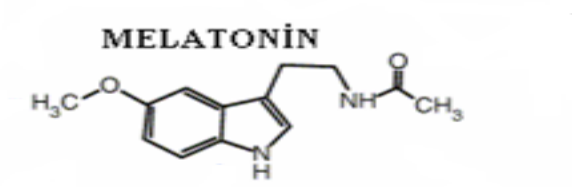

Figure 2. Chemical structure of melatonin.

Melatonin is a chronobiotic substance that plays a role in the regulation of many biological functions such as sleep, reproduction, circadian rhythm and immunity, as well as synchronizing body rhythms (9). It transmits information

about the duration of night to the circadian and circular systems. Thus, it acts as a clock and calendar in the body, providing information about the time of day and year, respectively, to the organism. This information is essential for sleep, temperature regulation, and seasonal reproductive changes (7).

Melatonin is also a powerful free radical scavenger (8). It is a powerful antioxidant and anti-apoptotic agent that prevents oxidative and nitrosative damage to all macromolecules, due to its ability to reduce the formation of reactive oxygen derivatives and reactive nitrogen derivatives, and directly excrete toxic oxygen derivatives formed in metabolic activities. It also stimulates the expression of antioxidant enzymes, immune system and anti-inflammatory genes that protect cells and organisms against bacterial and viral infections (10, 11). In addition; Melatonin and analogs that bind to melatonin receptors are important because they are used in the treatment of depression, insomnia, epilepsy, Alzheimer's disease, diabetes, obesity, alopecia, migraine, cancer, immune and heart disorders. In these cases, melatonin levels are usually low (9).

1.2. Mechanism

Melatonin secretion is correlated with nighttime length in many species. Melatonin secretion takes longer the longer the night is (12). The dark information received by the photoreceptors in the retina is transmitted to the suprachiasmatic nucleus (SCN) in the hypothalamus via the monosynaptic retino-hypothalamic pathway. This structure works autonomously, centrally and circadianly (13, 14). The impulses coming here then pass to the paraventricular nucleus (PVN), and the impulses received by the preganglionic adrenergic nerves of the sympathetic nervous system reach the pineal gland via the superior cervical ganglion (SCG). Sympathetic nerves secrete norepinephrine at the terminals on the parenchymal cells of the pineal gland rhythmically according to daily dark-light changes. This substance is recognized by β-adrenergic receptors in the pinealocyte membrane, accordingly the cyclic nucleotide system is stimulated and melatonin synthesis begins (15, 16).

In the pineal gland, the most important transmitter in the postganglionic sympathetic nerve endings is norepinephrine. During the day and in light, the nucleus suprachiasmaticus effectively stops the release of norepinephrine from these nerve endings. In the dark, norepinephrine is released from nerve endings and then norepinephrine binds to β-adrenergic receptors on the pinealocyte membrane. With the stimulation of β-adrenergic receptors, first adenylate cyclase is activated in the cell and cAMP increases. Then NAT and thus melatonin

synthesis increase. As norepinephrine release stops during the day or in light, cAMP and NAT levels rapidly fall (17). Melatonin concentration levels in the blood and cells at night are 3-10 times higher than during the day (3).

1.3. Metabolism, Mechanism of Action, and Excretion

Melatonin, which is highly soluble in water and lipid, quickly reaches all tissues of the body by passing directly through cell membranes to interact with intracellular receptors. In addition, melatonin performs its functions through specific membrane-bound receptors (6). These receptors are at least two high-affinity G protein-coupled receptors, MT1 and MT2 (18, 19). In addition, the MT3 receptor has recently been detected in hamsters. (11) MT1 receptors are localized in the biological clock, the SCN, and have also been detected in many other organs, including testes, ovaries, and leyding cells. MT2 receptors are mainly found in the brain, but are also present in the myometrium, granulosa cells, and testis. The MT3 receptor has been detected in liver, kidney, brain, oocyte and ovary (7).

Melatonin in the blood is 60-70 % bound to albumin. The half-life of melatonin is between 3 and 45 minutes. After being released into the bloodstream, it access to various body fluids, tissues, and cellular compartments (saliva, urine, cerebrospinal fluid, pre ovulatory follicle, semen, amniotic fluid, and milk). Since melatonin cannot be stored in the pineal gland, the plasma hormone profile provides a good indication of pineal function (20).

Melatonin is primarily rapidly converted in the liver to 6-hydroxydopamine, then through a series of reactions to N-acetyl-5-methoxy-6-hydroxytryptamine, and then to 6-sulphatoxymelatonin (or 6-hydroxymelatonin sulfate) conjugated with sulfate or glucuronide, and excreted in the urine. The excretion of 6-sulphatoxymelatonin in the urine is closely related to the serum melatonin concentration (3). At the same time, melatonin level in saliva has been accepted as an indicator of pineal functions in recent years (21). About 1% of melatonin remains unchanged in the urine (20).

2. Melatonin's Role in Reproduction of Small Ruminants

2.1. Melatonin and Photoperiod

Photoperiod is the main environmental variable used to time the annual reproductive cycle in small ruminants. Reproduction is controlled by an endogenous rhythm that inhibits for long photoperiods and activates for

short photoperiods (22). Thus, the main role of the photoperiod in the natural environment appears to be the synchronization of this endogenous rhythm. Photoperiodic information is processed by a complex neural and endocrine pathway to modulate reproductive activity (23, 24). Photoperiod stimulations are converted by the pineal gland into melatonin as a hormonal signal, Therefore, melatonin acts as a timer, allowing the animal to follow seasonal changes in the light/dark ratio (25).

In sheep, the secretion and plasma concentration of melatonin are low in daylight (19). After sunset, melatonin secretion increases 10-20 times and rises rapidly to reach a peak by the end of the night. Thus, the melatonin signal reflects the duration of the dark phase (25, 26). Changing the photoperiod conditions changes the amount and duration of the melatonin signal. Photoperiod stimulations control the release of GnRH hormone through the MT1 receptor (27). Decreased day length provides a secretion of melatonin that stimulates reproduction. In sheep with sustained and slow-release melatonin implant, the stimulating effect of short days is imitated and they enter the reproductive cycle earlier (28). Evidence of the effect of melatonin on seasonality comes from a study in which infusions of melatonin in pinealectomized sheep at doses mimicking long or short days elicited the same reproductive responses as nonpinealectomized sheep (26).

2.2. Melatonin and Estrus

In sheep and goats, melatonin sets off a cascade of processes that begin the mating season (29). Reproductive activity in sheep and goats living at mid and high latitudes varies seasonally throughout the year. Photoperiodic information is transmitted through changes in daily secretion of melatonin, its target receptor, in many tissues in the organism (30, 31).

Melatonin secretion, which increases with decreasing light exposure time in autumn-winter months, acts on the hypothalamus in sheep, stimulates GnRH secretion and initiating estrus (32). This occurs when the hormone melatonin stimulates GnRH and LH secretion by decreasing tyrosine hydroxylase activity and thus dopamine secretion during anoestrus. Because the dopaminergic system plays a role in the suppression of LH secretion by the estrogen hormone during seasonal anoestrus (33). In small ruminants many studies have focused on the role of melatonin in seasonal reproduction and exogenous applications for control of reproductive activity (34). On long days, sustained slow-release melatonin implants have been shown to advance the onset of the breeding season

in sheep and goats by mimicking the stimulating effect of short days (35, 36). The use of melatonin implants along with the ram effect simultaneously reduces seasonality in sheep and causes estrus to begin earlier than in sheep without (27, 37).

3. Conclusion

It has been reported that the hormone melatonin, which has been intensively studied since its discovery in 1958, has many physiological effects according to the living thing and tissue it affects. It is widely used in the field of human health due to its effects on jet-lag, antioxidant, antitumoral and mood disorders, especially through the circadian rhythm that changes depending on the change of daylight. Again, its critical role in the initiation of the reproductive cycle in sheep and goats has made a great contribution to its use in synchronization studies in the veterinary clinical field and thus to increase the yield in small ruminant breeding.

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CHAPTER IV

OVERVIEW OF THE GUT MICROBIOTA IN CAT AND DOG

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1. Introduction

The gut microbiome produces various metabolites, and these play roles in immune and metabolic pathways. All the microorganisms living in the organism are called "microbiota", and the genome carried by these microorganisms is called "microbiome" (1). The term "microbiome", introduced by the Nobel laureate Joshua Lederberg, refers to the collective genomes special to microbes or microbiota (2, 3).

Metagenomic sequencing of the next-generation sequencing technology has caused progress in the study of the microbiome. Metagenomic sequencing of V3-V4 variable regions of 16S gene rRNA has been used to study uncultivated gut microbial communities (4). The 16S rRNA gene has been a mainstay of array-based bacterial analysis for decades. The 16S rRNA gene consists of highly protected areas (5).

The gut is the largest endocrine organ in the body. Although fungi, protozoa and viruses are found in the composition of the gut microbiota, bacteria dominate the environment (6). Intestinal bacteria influence the health of the intestine and

other organs, including kidney, brain, and heart (7). Gut microbiota interacts with gut hormones and gut microbiome play great role in gut-brain crosstalk (8).

The gut microbiome is mainly located in the large intestine and consists mostly of facultative anaerobic bacteria. The four main phyla found extensively in humans are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (9-11). In dogs and cats, the dominant phyla are Firmicutes, Fusobacteria and Bacteroidetes and a lower proportion of Proteobacteria and Actinobacteria (12-15). The phylum-level distribution for cats and dogs is shown in the figure-1) presented by Hashimoto-Hill and Alenghat (16).

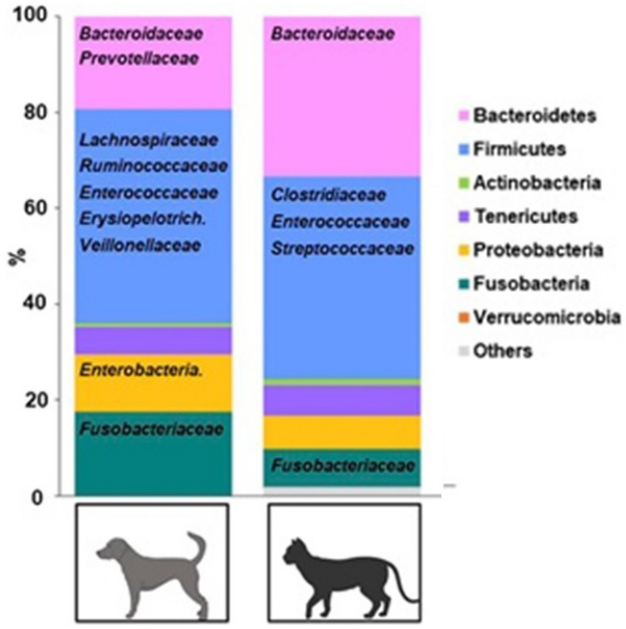

Figure 1. Fecal microbiota of healthy dogs and cats. Phyla are represented by color and bacteria (family level) are represented below the corresponding phylum (16).

Fusobacteria are abundant in the healthy gut microbiome of dogs, unlike microbiomes in humans and other animals (17). In humans, the presence of this phylum is associated with gastrointestinal diseases (18). This reveals that microbial differences play different roles in diseases of humans and dogs (19). The gut microbiome is involved in many metabolic events and changes in

the microbiome, and they play role in the pathogenesis of some diseases. For example, in obese dogs' gut microbiome, there is an increase in Firmicutes (Blautia, Peptoclostridium) and a decrease in Bacteroidetes (Bacteroides spp) (20).

The gut microbiome give metabolic responds and changes according to dietary components such as fiber, starch, fat, and protein. Changes in microbial communities also cause differences in metabolites that are important for health and produced by the gut microbiome. Therefore, the addition of prebiotics and probiotics to the diet of patient dogs may be beneficial in improvement the microbial diversity and normalization of the metabolism (21, 22).

2. Healthy/Unhealthy Gut Microbiome

Defining a "healthy" microbiota is difficult due to individual differences in microbiome composition and function. However, microbial diversity and stability are often considered as the key indicators of gut health, and they are inversely associated with chronic diseases and metabolic dysfunctions (23). Reduced microbial diversity has been shown to be associated with a variety of diseases. And an imbalance in the gut microbiota associated with unhealthy outcomes can be defined as dysbiosis, the result of many factors such as diet, antibiotics, stress, diseases, etc. (24, 25, 19). Dysbiosis is the loss of beneficial microbial community or signal and the increase of pathogenic microbes (pathobionts). And also dysbiosis is thought to trigger pro-inflammatory cytokines releases and immune dysregulation in various inflammatory diseases (26).

In addition to dietary components, the use of some drugs also leads to changes in the gut microbiome. For example, after using omeprazole, the microbiota returns to normal in 1-2 weeks (27). Also dysbiosis results from the use of antibiotics such as metronidazole and tylosin, and the gut microbiome returns to normal in 2-4 weeks after the use of these drugs. Sometimes this period may takes 1-2 months and may even be permanent (28).

Dysbiosis index (DI) is used to determine microbial dysbiosis in cats and dogs. This assay is the only analytically validated assay used to assess the stool microbiome and is a quantitative PCR-based assay. The DI reveals the total bacterial abundance as well as the fecal abundance of seven bacterial taxa, calculating reference ranges for these bacterial groups and a single number expressing the degree of intestinal dysbiosis. DI correlates negatively with species richness (29, 30).

In addition to assessing diversity, DI also helps to estimate by looking at the abundance of Clostridium hiranonis, a bile acid converting bacterium. Secondary bile acids are antimicrobial and suppress potential enteropathogens such as C. difficile, C. perfringens and E. coli. Therefore, the decreased abundance of C. hiranonis is strongly associated with fecal dysbiosis. The reason for the decrease in C. hiranonis abundance may occur commonly in exocrine pancreatic insufficiency and chronic enteropathy or after antibiotic administration (31, 32).

3. Gut Microbiome Function

The colonic microbiota plays a role in vitamin synthesis (vitamin K, and vitamin B group including biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin, and thiamine), intestinal motility, enterohepatic cycling of bile acids, and cholesterol metabolism (Figure 2) by direct and indirect actions (33, 34). The enzymes required for the synthesis of these vitamins by the gut microbiota are not encoded by the human genome (35).

Different species of gut microbiome play roles in vitamin synthesis. Magnúsdóttir et al. (36) systematically searched the genomes of 256 gut bacteria and they identified for their relation in the synthesis of eight B vitamins: biotin, cobalamin, folate, niacin, pantothenate, pyridoxine, riboflavin, and thiamine. Some of these genomes are involved in the synthesis pathway of all eight vitamins, while others contain none (36, 37).

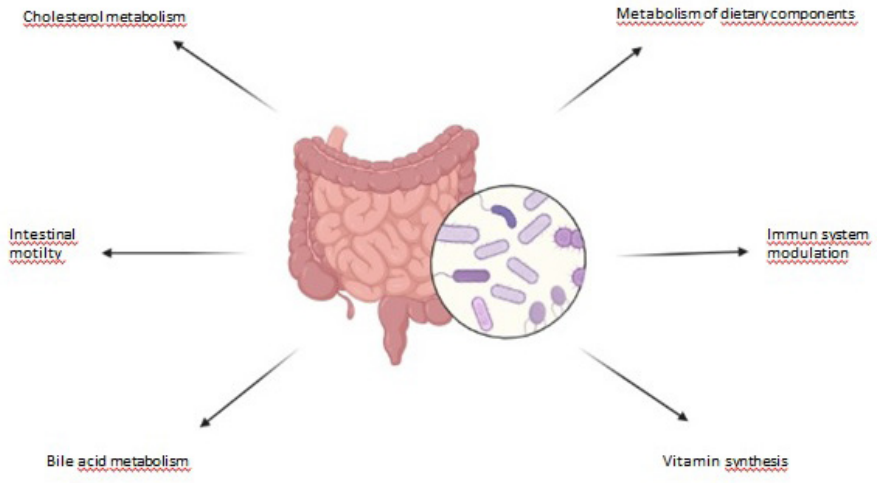

Figure 2: The gut microbiota functions.

Bacteria in the lower intestine play role in cholesterol metabolism. Its convert's performed cholesterol to coprostanol. It is poorly absorbed by intestinal mucosa, unlike cholesterol, and wastes the most abundant sterol with feces (38). This conversion is predominantly carried out by the taxa Lactobacillus and Eubacterium (Eubacterium coprostanoligenes). The positive side of this transformation is that it reduces the risk of cardiovascular disease. However, the enzymes responsible for this conversion are unknown (39, 40).

Impaired with the disruption of the synthesis of secondary bile acids by gut microbiome, and interaction between intestinal bacteria is associated with various disorders such as Clostridioides difficile or Salmonella Typhimurium infection, inflammatory bowel disease, Type 1 diabetes, asthma, metabolic syndrome, obesity, Parkinson's disease, schizophrenia and epilepsy (40).

One of the most important tasks of the intestinal microbiota is also to synthesize short-chain fatty acids (SCFA). Prebiotic nutrition is needed for the gut microbiome to have a healthy diversity. A prebiotic is defined as a "non-digestible compound that modulates the composition and/or activity of the gut microbiota by being metabolized by microorganisms in the gut, thereby conferring a beneficial physiological effect on the host" (41). Prebiotics are classified as fiber, which is the indigestible part of plants, but not all fiber is prebiotic (42). Dietary fiber escapes enzymatic digestion in the small intestine and reaches the large intestine relatively unchanged. Dietary fiber includes both soluble and insoluble forms (43). Soluble dietary fibers are easily fermented by the gut microbiota and are important for a healthy gut environment and metabolic homeostasis (44). The product of this fermentation is short chain fatty acids (SCFA), mainly acetate, propionate, and butyrate (45). It also affects SCFA produced due to the direct effect of dietary composition on the fecal microbiota. For example, a high protein diet promotes the growth of butyrate-producing bacteria in dogs regardless of body condition. However, fecal microbiota in lean dogs is more resistant to dietary protein changes than obese dogs (46). In addition, SCFA provides approximately 10% of the host's daily energy needs (47).

Colon cells use butyrate as their primary energy source (predominantly butyrate-producing bacteria Roseburia spp. and Eubacterium rectale). Butyrate is vital for colonocytes barrier function. Propionate (produced by Bacteroidetes species) first enters the portal circulation. It is then used in gluconeogenesis in the liver and takes part in the formation of hunger and satiety signals. Acetate is circulated in various peripheral tissues (43, 48-50).

An increase in Prevotella and acetate is observed with rye added to the diet of dogs at a rate of 50%. An increase in Prevotella has been associated with stability in glucose metabolism as in humans, and it is thought that by increasing acetic acid it may suppress appetite in dogs as well (51).

SCFAs also support potentially beneficial bacteria such as Bifidobacterium and Lactobacillus by lowering the pH in the colon (52, 53). On the other hand, it also restricts the proliferation of bacteria associated with gastrointestinal disease in dogs, such as Clostridium perfringens and Escherichia coli (54, 55).

Clinical studies in humans have revealed decreased fecal concentrations of SCFAs in patients with chronic GI disorders such as inflammatory bowel disease and colorectal cancer (56). More common in animals with inflammatory bowel disease than in those without the disease, dysbiosis is characterized by a reduction in microbiome diversity in general and a reduction in SCFA-producing species. There is an increase in Enterobacteriaceae and E. coli bacteria attached to the epithelium or invading the intestinal mucosa in ileal and colonic mucosal microbiota samples from dogs with IBD (57). On the other hand, dogs with chronic enteropathy have significant changes in the stool microbiota and an altered stool SCFA concentration (58).

Studies on the use of probiotics and prebiotics in some gastrointestinal diseases such as diarrhea, inflammatory bowel disease, and irritable bowel syndrome are ongoing (59). Probiotics, which are used to improve the gut microbiome and the health of the host, have been defined as live microorganisms that can provide health benefits to the host when consumed in adequate amounts as part of a food (60). Most of the probiotics studied in pet health belong to the genera Lactobacillus, Bifidobacterium, and Enterococcus (61-64).

The addition of probiotics to the diet in dogs resulted in an increase in the concentration of acetate and butyrate in feces and a decrease in ammonia. It also enhances cell-mediated immune responses to antigenic challenges (65-66). Prebiotics are easily added to the diet and can be found in the daily diet. Probiotics can also be added to the diet to help support bacterial populations and certain disorders that affect gut health. The use of probiotics is a temporary process based on need. However, unlike some other drugs and antibiotics, no harmful effects were observed in long-term use of prebiotics or probiotics (67).

4. Conclusion

The gastrointestinal microbiome of cats and dogs is increasingly recognized as a metabolically active organ inextricably linked to pet health. There is growing

research that suggests that dietary components not only cause GI disease, but can also affect allergies, oral health, weight management, diabetes, and kidney disease through changes in the GI microbiome. The use of probiotics should be reconsidered, given the fact that there are individual and species differences in microbiome composition. On the other hand, there is a need for new studies on the use of probiotics and prebiotics as well as the development and regulation of the GI microbiota.

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CHAPTER V

A PRELIMINARY OVERVIEW: INVESTIGATION OF THE POSSIBILITIES OF CONSTITUTING A MUTUAL MORPHOLOGICAL NOMENCLATURE METHOD IN TURKISH INDIGENOUS PIGEON GENOTYPES

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1. Introduction

The domestic pigeon originated from the rock pigeon (*Columba livia*). It is assumed that domestication was implemented in the Mediterranean region about 5.000 years ago (1,2). Domestic pigeons have been artificially selected by the breeders for many traits, including cranio-facial structures, vocalizations, plumage color, color patterns, flight performance, and aerial-display characteristics (3-8). For plumage color variation in pigeons, Domyan et al. (9) stated that "Artificial selection over the centuries has produced a wide variety of feather pigmentation in rock pigeons, with the combined effects of dozens of loci." Undoubtedly, changes in the lifestyle of the human population, and socio-economic changes play a role in the distribution of pigeon phenotypes (10).

Urbanization generates significant changes in habitats and creates new urban habitats affected by human activities (11,12). This has led to changes

in some morphological and behavioral characteristics in pigeon populations. Some phenotypes are more common in urban areas than rural areas, indicating that purposive selection has occurred in urban habitats (11,13-15). Blue colored pigeons are more frequently in rural areas and melanic pigeons (darker plumage color) are more freqently in city centers (10,15) Today, it is stated that there are more than 350 pigeon breeds with different morphological and behavioral characteristics (2,7,8). Aoussi et al. (16) drew attention to behavioral changes in birds living in the urban-habitat with the term "urbanization syndrome". Although the basic molecular, biochemical and physiological mechanisms for the formation of plumage colors in pigeons are known, there are still unexplained dark spots. This situation was summarized by the researchers as follows: "We now better understand how different types of pigment are determined, but we know much less about how these pigments are deposited to generate color patterns within and among feathers" (17,18).

As there are so many genetic and environmental factors in the formation of morphological traits, it is very difficult to classify pigeons according to these traits. Although pigeon breeds are classified according to their body structure, body shape, color and pattern of plumage pigmentation in other parts of the world, they are often classified according to their flight display characteristics in Turkey. The breeds of pigeons in Turkey are classified into nine main groups: diver (*dalıcı*) (Azman, Bango, Baska, Dewlap, Dolapci, Domino, Donek, Kelebek, Misiri, Oynak, Spotted Kelebek, Şarabi, and Yoz), tumbler (*taklacı*) (Alabadem, Anatolian, Gogsu ak, Iç aglı, Ketme, Kizilbas, Taklambac), roller (*makaracı*) (Bursa roller, Cakal, Corum, Smyrna roller, Mulakat, Oriental roller, and Thrace roller), spinner (*dolap dönücü*) (Telkuyruk and Trabzon), fleet flyer (*filo uçucusu*) (Abu abse, Amberi, Bagdat, Bastankara, Bayramli, Buludi, Burmali, Cici, Damascene, Gullu, Halebi, Scandaroon, Ispir, Karakuyruk, Kespir, Kullu, Msawad, Shicki, Shafari, and Sirtikizil), high flyer (*yüksek uçucu*) (Katal, Van highflier, and Yasmakli), racing/homer (*postacı*) (Homer racing), ornamental/show (*form/süs*) (Gumuskuyruk, Hunkari, Karakan, Selcuklu, and Tavuskuyruk), and singer (ötücü) (Ankut trumpeter, Turkish whisperer, Demkesh, and Kumru trumpeter). The Dolapci, Donek, Kelebek, and Oynak pigeons can be further classified as the spinner, and the Alabadem, Gogsu ak, Iç aglı, Ketme, Kizilbas, and Taklambac pigeons are also known as show birds (19).

Over time, ornamental features have come into prominence pigeon breeds and genotypes such as Alabadem, Bango, Baska and Mısıri. Therefore, some

indigenous pigeon genotypes and breeds can be included in both classifications (Table 1 and 4). Pigeon genotypes bred in Turkey may have mutual traits among themselves, as well as distinctive traits. Baska, Bango and Mısıri pigeon genotypes have short-beak structure, and prominent cere structure as mutual traits. However, of these three genotypes, Bango and Mısıri pigeons are similar to each other. The Mısıri pigeon differs from the Bango pigeon with its frilly feather structure on the chest (rosette) (Table 4). Moreover, the nomenclature of the common morphological characters in indigenous pigeon genotypes differs according to the regions of Turkey. Since it is a traditional breeding branch, morphological features can be named in local terms. This book chapter, it is aimed to compile information from the studies that pioneered the determination of the morphological characteristics of Turkish indigenous pigeon breeds and genotypes. It is also aimed to investigate the possibilities of constituting a mutual morphological nomenclature method that can provide clues for future studies.

2. Plumage color

Plumage colors and color patterns have a major impact on mate selection, camouflage, recognition, and communication in birds (8,20). Melanin is the most common color-producing pigment, with a wide-spectrum of black, brown, and gray. There are two chemical variants of the melanin, eumelanin (black) and phaeomelanin (rusty-red or rufous). Both types are produced by organelles called melanosomes in melanocyte cells in the deepest layer of the skin, stored and transferred to keratinocyte cells (20,21). The various hues result from differences in the amounts of the two types of melanin (eumelanin-brown/black) and (pheomelanin-yellow/red) (22). Most melanin-based feather colors are produced by a mixture of the two in varying concentrations of eumelanin and phaeomelanin eumelanin, and phaeomelanin pigementation is easily distinguished morphologically (20,23). The blue/black and brown-appearing phenotypes have feathers containing high amounts of eumelanin and low amounts of pheomelanin pigments, while phenotypes with an ash-red (recessive red) appearance have feathers containing low amounts of eumelanin and high amounts of pheomelanin pigments (9,22,24). On the other hand, carotenoid pigments are produced by biochemical altering carotenes in diet and deposited in fat globules within the feather cells. Produce bright red, orange, and yellow colors (20,25,26). Shawkey and D'Alba (20) stated that color reproduction is not only dependent on pigments (melanin, carotenoid). They emphasized that it is formed by various pigment molecules, nanostructures (keratin), or a

combination of both. Shawkey and D'Alba, 2017 called this situation "structure-pigment combination". The iridescent green color can be produced in feathers by the harmonious scattering of light by layers of keratin and melanin. (20). An example of this is the iridescent neck blend color (*green, purple, green-purple*) of the Bursa oynarı pigeon (27). We can observe the reflective green/reflected green-purple feathers on the necks of black, smoky, and sky (*blue*) plumage colors in Ankara tumbler pigeons, scarlet plumage color in Alabadem pigeons, black, red, and ashy (*smoky*) plumage color in Oriental pigeons, and the blue plumage color in Mulakat pigeons. The same reflected-feathers can be observed in Cins pigeons (*Adana or küpeli*) with blue plumage color (5,6,28,29).

The non-reflective blue color is generated by co-localized melanomas (melanin-filled organelle). These melanin-filled organelles are typically found at the basal spongy layer and are critical in absorbing scattered white light. Only a certain part of the light is scattered by the spongy layer to produce blue, and the remainder is scattered inconsistently. Without the light absorption provided by melanin, this white color illumination suppresses the blue, resulting in a whitish-blue color (20,30). We can observe non-reflective light and dark blue plumage colors in many indigenous pigeon genotypes in Turkey. Examples of the genotypes with this color are light and dark blue (tile-blue) plumage color in the Mulakat pigeons, and blue body color in the Baska pigeons (Table 2 and Table 4). In addition, the body color of different shades of blue in Ankara tumbler pigeons is also defined as the blue (*gök*) (28). Melanosomes located on or inside the keratinous spongy layers prevent light from reaching them, resulting in a black color. In some cases, several spongy layered cells surround a single layer of melanosomes. However, how this affects color has not been investigated (20). We think that different shades of black can be seen depending on the case that the spongy layer-cells are surrounded by melanosomes as single-layer and multi-layer. Black plumage color is a very common trait in Turkey indigenous genotypes. Some breeds or genotypes in which this color is determined: Bursa oynarı, Thrace roller, Oriental, Muradiye kelebek, Ankara Tumbler, and Squadron flyer pigeons (6,27,28,31,32) (Table 1,2,3). In addition, we think that the shiny (*yanar-döner*) plumage color found in Ankara may be different shades of black plumage color (28).

3. Chocolate-brown, and pale-yellow

Chocolate-brown (çikolata) plumage color is a character found in Baska, Bango and Mısıri pigeons (Table 4). Brown plumage can often be mistaken for dun

(dilute black). The true dun color refers to dilute spread of black pigmentation, while the chocolate-brown color refers to intense spread of brown pigmentation. In case of intense spread, it appears as a chocolate-brown color. In dilute spread, it appears as a very pale yellowish color (33). We think that the ligh-yellow color is described as citrin (*sarı-alı*) in Alabadem pigeons (5), and as lemon-yellow (*limoni*) in Bango, Baska and Mısıri pigeons (Table 4) . Brown plumage color often fades with the advancing age (33). Özbaşer et al. (6) stated that the red feathers become lighter with advancing age in the Cakal pigeons. Shawkey and D'Alba (20) emphasized that feather color in birds may change over time depending on moulting. We think that the reason why the body color of the Cakal pigeon becomes lighter with time is the 'moulting'.

4. Head plumage, and head marks

Head plumage in the Bursa oynarı pigeons was examined by dividing it into five groups as markless, scarfed, speckling, browed and mottled (head marks in Table 1) (27). In the Mulakat pigeons, this situation was evaluated as a head marks, and named as T-pattern (6) (Table 2). It has been reported that there is an irregularly-shaped mark (Almond-*badem*) on the upper part of the head in the identical color as the plumage color of the body in the Alabadem pigeon (5) (Table 1). It is an important trait in Cakal pigeons that the eye ring is distinctly lighter than the plumage color. Oriental pigeons with speckled plumage color have white-irregular small patches on the head (Table 2). In the Baska pigeons, on the other hand, it is a desirable trait that the head is covered with white feathers (white-head) (*akbaş*). The presence of a straight uninterrupted (straight mark line) or curved-dashed line (camber mark-line) at the level of the lower mandible, that separates the white-head feathers from the colored-body feathers is a genotype-specific character (Table 4). The presence of black pigmentation around the eyes in Squadron flyer pigeons is called "*Karagöz*". This pigmentation encircles the entire eye in the form of a ring (32).

5. Eye color

The color of the eye is represented by iris color, which is the result of both pigment deposition and structural coloration. Pigment cells are found in the stroma layer of the eye. The diversity of eye colors in birds depends on the deposition of multiple non-melanin pigments (pteridines, purines, and carotenoids) in the iris. Eye color in birds varies according to the presence or

absence of pigment cells in the iris and the content of pigments in these cells and exhibits variations, ranging from black, dark-brown to white, yellow, and orange (34,35). The most common iris colors in domestic pigeon are white (pearl), orange and bull (dark brown). However, the orange eye color are seen in shades ranging from yellow to red depending on the density of the blood vessels. Bull-eye color mostly observed in white plumage birds, and also known as "odd eyes" (34-38). Si et al. (35) emphasized that the pearl-eyed trait in pigeons emerged with domestication, and it was formed by the outcome of artificial selection. The white-eye occurs due to the limited deposition of multiple non-melanin pigments (pteridines, purines and carotenoids) in the iris. Bull-eye is the name given to the dark brown pigmentation of the iris in birds. Pigeons have two types of non-melanin pigments in their iris: guanidines and pteridines guanidines are albescent (whitish) opaque pigments, and pteridines are yellow-orange pigments. Pigeons with orange eyes have both guanidine and pteridine pigments in the iris stroma. Pigeons with white eyes have only guanidine pigment in the iris stroma. (34,37,38). The situation in pigeons with bull eyes is due to the absence of white and orange pigments in the iris stroma, so the dark melanin pigment underlying the iris pigment epithelium gives the eye its color (34,37-39). Eye colors ranging from white to bull color are determined in Turkey's indigenous pigeon breeds/genotypes. White eye color was determined in Bursa oynarı, Cakal, Oriental, and Muradiye dönek pigeons, and bull eye color was determined in Baska, Bango and Mısıri pigeons. The eye color called Rose (dusty-rose) is seen in different shades of red in Bursa Oynarı, Cakal, and Mulakat pigeons (Table 1,2,3,4).

6. White feathers on some regions of the body

White feathers can be seen on the rump, under the tail, wing primaries, and tail feathers (40). While these white feathers appearing on certain parts of the body are defined as 'mark (*nişane*)' in some studies (28,32) they are included in the plumage color according to some studies (5,31,41). The presence of white feathers on the wing primers of Ankara tumblers is defined as 'brooken-color wing (*kır-kanat*)'. In the same research, some of the tail feathers are white, 'white tail (*ak-kuyruk*)' , and the presence of white feathers under the tail is defined as ' mirror (*ayna*)' (28). In Squadron flyer pigeons, the presence of white primaries on the wing was described as 'shove (*sokuşturmalı or arans*)', while the tail consisting entirely of white feathers is described as 'silver tail (*gümüş-kuyruk*)'

(32). This situation is shown as a distinctive breed character in Bursa oynarı pigeon (black pied coloration; black wing white tail or white wing-white tail) (27) (Table 1). The same situation is likely to be a distinctive breed character in Cakal pigeons (white-wing white tail) (6) (Table 2). Pigeons with white feathers on the wings, tail, or other parts of the body in addition to the plumage color were called 'jackal (çakal)' in Thrace roller pigeons (31). Alabadem (5), Cins (*Adana or Küpeli*) (29), Bango and Mısıri pigeons also have white feathers on their wing primaries, and in the tail feathers (Table 4).

7. White-side pigeons, are the Bango and Mısıri color-sided pigeons?

Walker (40) drew attention to white-side pigeons, and described these pigeons as the white color covers the entire wing except the primaries. It is especially occurs in yellow and red birds. In Bango and Mısıri pigeons, the body color is white, in some individuals the red or yellow color covers entire the wing, except the primaries. This situation is called as red-wing (*kırmızı-kanat*) or yellow-wing (*sarı-kanat*) (Table 4). We think that this character in Bango and Mısıri pigeons can be called 'color-sided'.

8. Speckled

Another character is speckled, 'grizzle' by another author's definition, which is described by the Turkish fanciers as 'tiger (*kaplan*)'. This character is often observed in Oriental pigeons as black-mottled (black tiger /siyah kaplan), red-mottled (red tiger/kırmızı kaplan), and gray-mottled (gray-tiger/*gri-kaplan*) (6,40,41). This condition is known as 'kesper (*keşpir*)' in Squadron flyer pigeons, and according to the main body plumage with white mottles on it, it is called black-kesper (*siyah-keşbir*), red-kesper (*kırmızı-keşbir*) and blue-kesper (*mavi-keşpir*) (32). Breeders define the speckled trait in Muradiye kelebek pigeons in different ways, which are the *'şeş, kaplan-şeş* or *bovma*' (Table 3).

9. Beak pigmentation

Walker (40) stated that birds with almost nearly-white plumage color have a dark beak or stained beak. Özbaşer et al. (41) defined this trait as the appearance of black spots (*zikir*) on the beak and claws of some Muradiye dönek pigeons (Table 3). Beak pigmentation (*zikir or zikirli*) has also been reported in some Thrace roller pigeons (31).

10. The cere

A ring of bare skin, which can vary in color depending on the species, surrounds each eye (orbital ring or eye cere). Also, some Pigeons have a slightly swollen naked area surrounding the nostril, which is called the cere. In Dragoon pigeons, the nose and eye cere structure can be observed as swollen and exaggerated structure. Some carrier pigeons (especially English carrier pigeons) have a carunculated-cere, while Black Spanish Barb pigeons have an outgrowth eye ring (42). Stettenheim (26) described 'the cere' structure as partly feathered in many parrots, but bare and often brightly in other parrots, pigeons, falconiformes, owls, and some cradics. Bango, Baska, and Mısıri pigeons have prominent eye and nasal cere. It is defined as a small integumentary outgrowth structure with a powdery appearance and a lime-white color (Table 4).

11. Head Crest

Head crests of birds are found in many bird species and, it serves important functions in coutrship and social displays and mate selection (2,18,44,45). The head crest is the name given to the growth of a group of feathers containing head and neck feathers with opposite polarity. Researchers proved that the head crest structure was associated with a single nucleotide coding mutation (intracellular kinase domain of *EphB2*) (7,46). Peak crest, shell crest, mane and hood can be observed commonly in pigeons. The feathers that composed of the head crest can be small and few in number as on the peak crest or many and ostentatious as in the hood crest. While the Indian fantail pigeon breed has a peak crest, the Old german owl pigeon breed has a shell crest structure. Old dutch capuchin breed with mane structure, and Jacobin pigeon breed with hood structure can be given as examples of crested pigeons (7). Head-crest was determined in Alabadem breed (5) and Muradiye kelebek genotype, which are indigenous pigeons of Turkey (Table 1,3).

12. Feathered-tarsi (feathered-feet)

In the rock pigeon (*Columba livia*), from which the pigeon breeds are derived, the tarsi are naked (42). A study conducted in 2016 found that a gene called *Pitx1* is expressed at lower levels in the developing legs of feathered feet breeds than in a breed with scaled-feet. Experiments also showed that *Tbx5* was abnormally expressed in the embryonic hind limbs of feathered-feet pigeon and chicken breeds (47). In a recent study, the feathered-tarsi were examined

in four groups: scaled (naked), grouse, small-muff, and large- muff. Old Dutch Capuchin and African owl pigeons have naked tarsi (scaled), while the tarsi of English trumpeter, Fairy swallow pigeons are covered with long feathers (47). The Muradiye kelebek pigeons have feathers extending down from their metatarsus and covering certain digits, and it is called "grouse" (Table 4).

13. Short, medium, and long- faced pigeons

It has been reported that beak morphology is related to cranial morphology in bird species (48,49,50). For this reason, short-beaked pigeons are also known as short-faced pigeons. There is very limited information about short-faced pigeon breeds. Artificial selection has led to cranio-facial diversification in domestic pigeons. 'Short-face' pigeon breeds have reduced premaxilla or beak size, so they are often unable to feed their hatchlings and human intervention or surrogate parents are needed to feed the hatchlings (51). The most well-known short-faced pigeon breeds are Berlin short-faced tumbler, Budapest short-faced tumbler, English short-faced tumbler, and African owl pigeon breeds. The Vienna medium-faced pigeon breed can be given as an example of the medium-faced pigeon breeds, and the Berlin long-faced tumbler pigeon breed can be given as an example to the long-faced pigeon breeds (3,51). The Baska, Bango, and Mısıri genotypes, which diver and ornamental features come into prominence, are short-beaked pigeons (Table 4).

14. Plumage color, and wing pigmentation pattern (T-check, checker, bar or barless)

Darwin (52) suggested to classify domestic pigeon breeds according to morphological characteristics, and he classified 32 breeds into four groups: 1- the pouters and croppers (enlarged crops) 2- wattle breeds (elaborated beaks and the large-bodied runts) 3- artificial breeds (relatively short beak); and 4- Breeds that resemble the ancestral rock pigeon (3,52). Čanády and Mošanský (53) divided feral pigeons into seven groups according to their plumage phenotypes: 1- Blue-bar (wild): Bluish-gray plumage color, with two dark stripes on the wings, and stripes of the same color at the tip of the tail. Birds have a distinctive metallic green shiny on their necks, 2- Blanc (white or albino): straight white or almost white plumage, no stripes on the tail and wings, and a metallic iridescence on the neck, 3- Black (spread): straight black plumage, striped tail and wings, tail stripes invisible or absent, and in many pigeons a metallic iridescence on the

neck. 4- Checker (light-checker): Steel blue-gray plumage, two dark stripes on the wings, one dark stripe on the tail, green iridescence on the neck. A typical characteristic of this phenotype was the checkerboard-like spotting on the wings. 5- T-pattern (dark-checker): this phenotype resembled a "checker phenotype" form with a dark blue-gray color, dark spots on the wings was more abundant, so the spots might merge. Stripes on wings were poorly visible, and metallic iridescence on the neck, 6- Rufous: beige-and-gray plumage, sometimes with white elements, two brown stripes on the wings, no stripes on the tail, and metallic iridescence on the neck. 7- Others: included pigeon phenotypes not classified with any of these forms.

Guernsey et al. (54) classified the variation in plumage pigment domestic pigeons as eumelanic phenotypes (Black-check, blue-bar, brown, pheomelanic phenotypes (ash-red check, ash-red bar, yellow-check, recessive red) and White. In this classification, eumelanic pigeons were defined as birds with plumage color in shades of brown or black, including gray, and pheomelanic pigeons as birds with red or yellow plumage color (54). Domyan et al. (9) defined common eight different plumage colors produced by three loci as 'melanin phenotype', and they stated that these types are blue, brown, dun, khaki, ash-red, ash-yellow, recessive-red, and recessive-yellow. In the same research, ash-red, blue, brown, and recessive-red feather pigmentation is referred to as 'non-dilute', ash-yellow, dune, khaki, and recessive-yellow feather pigmentation as 'dilute'. Rock pigeons (*Columba livia*) fundamentally show one of the four basic color pattern phenotypes. In order of decreasing pigmentation (melanism): T-check, checker, bar, or barless. The barred phenotype is based on ancestral genes (8). Vickrey et al. (8) reported that the *NDP* gene plays an important role in wing pigmentation, and it is more expressed in the feathers of pigeons with checker and T-check phenotypes (darker patterns). In the same study, she emphasized that the lightest colored birds had a mutation in *NDP*, the gene that causes less pigmentation. She also added that the NDP mutation causes visual defects in pigeons (8). Wing pigmentation pattern were determined in some individuals of Alabadem (barred) (5), Baska (barred), Bango (barred), Mısıri (barred) (Table 4), Mulakat (barred), Oriental (barred), and Muradiye dönek pigeons (T-check, checker, barred, barless) which are indigenous breeds and genotypes in Turkey (6,41).

15. Conclusion

Pigeon breeds can be classified according to flight characteristics, plumage color, wing pigmentation patterns, and even craniofacial structure. It is obvious

that a wide variation in morphological characteristics in some indigenous pigeon genotypes in Turkey. However, it seems possible to classify according to morphological characteristics by defining all phenotypic features at an individual level. For the correct application of this classification, it would be appropriate to define the indigenous pigeon genotypes at the morphological and molecular levels. It is necessary to constitute a mutual nomenclature method by forming a consensus in the triangle of researchers, breeders, and breeders' associations. In addition, domestic pigeon genotypes should be registered with studies that give priority to genotypes with distinctive traits.

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Table 1. Morphological characteristics of Alabadem and Bursa oynarı pigeons.

Breed/genotype Registiration	Classification	Region	Eye color	Plumage colors	Head marks	Body marks	Head crest	Feathered-tarsi	Distinctive traits/marks	References
Alabadem (registered)	Tumbler Thracer Ornamental	Edirne and adjacent areas in Thrace (especially Uzun köprü and Havsa)	Black and greyish blue	Black, Red, Yellow, Chickpea, Scarlet, Citrin, Ashy	White-head, imprint almond, bolted almond	Wing pigmentation pattern in some birds (barred)	Crested	Scaled	White-head and almond-shaped head pigmentation	5,19,47
Bursa oynarı (Bursa roller, Bursa pigeon) (registered)	Roller Tumbler	Bursa and İstanbul provinces	White, dusty rose, dark dusty rose	White, black, White, Black Wing-white tail, White wing-white tail	Markless, browed, piebald, mottled, scarfed, speckling	White and black (ventral abdomen color); green, purple, green-purple (neck blend)	Uncrested	Scaled	Veil, Black pied, head and bib pigmentation, shiny black feathers, beak and claw pigmentation	27,19,47

Table 2. Morphological characteristics of Cakal, Mulakat and Oriental pigeons.

Breed/genotype Registiration	Classification	Region	Eye color	Plumage colors	Head marks	Body marks	Head crest	Feathered-tarsi	Distinctive traits/marks	References
Cakal (Çakal, Cakal roller, nalbant) (unregistered)	Roller	Tekirdağ, Aegean and Marmara region	Rose, white	Red (White wing-white tail)	Eye ring color distinctly lighter than plumage color	Wing tips and tail are white, other parts of the body are red	Uncrested	Scaled	Prominent eye ring, red plumage color, wing tips and the tail are white	6,19,47
Mulakat (Mülakat) (unregistered)	Roller	Tekirdağ, Bursa, İstanbul	Blue, light -dusty rose, dark -dusty rose	Light blue, dark blue, tile blue	Veil and T-pattern in some birds	Wing pigmentation pattern in some birds (barred)	Uncrested	Scaled	Veil, wing tips and the tail are white	6,19,47
Oriental (Oryantal, Ottoman thracer) (unregistered)	Roller	Tekirdağ, Balıkesir, Kırklareli (Lüleburgaz)	White, White with yellow speckled, white with red speckled	Black, White, red, ashy, yellow, and speckled	speckled plumage color white-irregular small patches on the head	Wing pigmentation pattern in some birds (barred)	Uncrested	Scaled	Spesific body posture*	6,19,47,55

* The Oriental pigeon has a specific body posture. The tail is withholding almost perpendicular to the horizontal line of the body in a normal posture, and the wings are kept below the horizontal line of the body, not touching the ground (55).

Table 3. Morphological characteristics of Muradiye dönek and Muradiye kelebek pigeons.

Breed/genotype Registiration	Classification	Region	Eye color	Plumage colors	Head marks	Body marks	Head crest	Feathered-tarsi	Distinctive traits/ marks	References
Muradiye dönek (Selanik, Muradiye) (unregistered)	Diver-spinner Diver	Balıkesir, (Muradiye district)	Black, white, blue	Black galaca, red galaca, blue galaca,	Black feathers at the level of the lover mandible in some birds	Wing pigmentation pattern (T-check, checker, barred, barless), tail-belt in some birds	Uncrested	Scaled	Beak and claw black pigmentation in some birds.	19,41,47
Muradiye kelebek (Kelebek, Saya) (unregistered)	Diver-spinner Diver Ornamental	Balıkesir, (Muradiye district) Nationwide	Grayish-blue, black	White, black, sable, spotted, jackal, black-tailed, yellow-tailed almond, band-tailed, blue, black-headed	In some birds, a small irregular-shaped feather pigmentation on the top of the head, the same as the body color.	-	Crested	Grouse	Irregularly- shaped patches of body color the same color as the tail, these patches were found mainly on the neck and under the beak.	19,47,56**

**56: NKU, Scientific Research Projects Coordination Unit, Project final report.

Table 4. Morphological characteristics of Baska, Bango and Mısırı pigeons

Breed/ Genotype Registiration	Classification	Region	Eye color	Plumage colors	Head marks	Body marks	Head crest	Feathered-tarsi	Distinctive traits/marks	References
Baska (Akbaş) (unregistered)	Diver Ornamental	İstanbul (Thrace and Aegean regions)	Black or dark brown (bull-eyed)	Black, red, yellow, chocolate blue, chickpea, lemon-yellow	White-head, straight or camber mark line	Wing pigmentation pattern in some birds (barred)	Uncrested	Scaled	White-head, Straight or camber mark line, short-beaked, prominent beak and eye cere	19,47,56**
Bango (unregistered)	Diver Ornamental		Black or dark brown (bull-eyed)	Black, red yellow chocolate blue, chickpea, lemon-yellow, white	Head and body covered with white feathers	Wing pigmentation pattern in some birds (barred)	Uncrested	Scaled	Short-beaked, prominent beak and eye cere	19,47,56**
Mısırı (Mısıri, Mısırlı, mısır, güllü) (unregistered)	Diver Ornamental	İstanbul Nationwide	Black or dark brown (bull-eyed)	Black, red, yellow, chocolate blue, chickpea, lemon-yellow, white	Head and body covered with white feathers	Wing pigmentation pattern in some birds (barred)	Uncrested	Scaled	Short-beaked, prominent beak and eye cere, frilly feather structure on the chest (rosette)	19,47,56**

****56: NKU, Scientific Research Projects Coordination Unit, Project final report**

CHAPTER VI

CRANIAL CRUCIATE LIGAMENT RUPTURE IN DOGS: A TECHNIQUE OF ADVACEMENT TUBEROSITAS TIBIA

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1. Introduction

The cranial cruciate ligament rupture technique, which is one of the main causes of common hind limb extremity lameness in dogs, has been widely used in recent years. Today, the prominent approaches in the repair of cranial cruciate ligament ruptures are; extracapsular techniques, techniques to eliminate the breaking force in the knee joint and techniques made by utilizing the biomechanics of the knee joint. The angle between a line drawn from the tibial plateau in the knee joint and the strut lowered from the patellar tendon is called the "patellar tendon angle" (PTA). Biomechanically, the load on the knee joint is zero when the PTA is at 90°. To ensure this angle is 90°, the tuberositas tibia should be moved forward. In the radiograph taken 135°±5° mediolateral of the femorotibial angle of the knee joint, the tuberositas tibia is moved forward in

order to make the PTA 90°. An osteotomy line is created in tuberositas tibia. The tuberositas tibia is moved forward by placing an implant called a "cage" that will enable the forward carrying process to the created osteotomy line. In this section, two different methods used in the treatment of cranial cruciate ligament ruptures were explained, which is one of the most important problems of the knee joint of dogs.

2. Anatomy of the Knee Joint

The function of the knee joint, which has a complex hinge-like structure, is the flexion and extension movement of the hind limb. (1-4) The primary function of the knee joint is through cranial cruciate ligament, caudal cruciate ligament, medial collateral ligament and lateral collateral ligament. (2,5) Cranial cruciate ligament, which is one of the intra-articular ligaments; It starts from the caudomedial of the condylus lateralis of the femur and extends cranially, medially, and distally, and adheres to the intercondylaris cranialis of the tibia (Figure 1). (1,6,7) This ligament controls the extension and cranial movement of the tibia. (8) The other ligament localized in the joint is the caudal cruciate ligament. (2) Caudal cruciate ligament; It attaches to the fossa on the lateral edge of the medial femoral condyle and passes caudodistally to the medial of the popliteal notch of the tibia. (1) This ligament controls the flexion and caudal movement of the tibia relative to the femur. (8) In addition, the caudal cruciate ligament bends together with the cranial cruciate ligament, limiting the internal rotation of the tibia. (9) The cruciate ligaments are dynamic and they are direct intra-articular limiters due to their anatomy and location. (2) Other structures in the knee joint are fibrocartilaginous semilunar medial and lateral menisci and meniscal ligaments. (10)

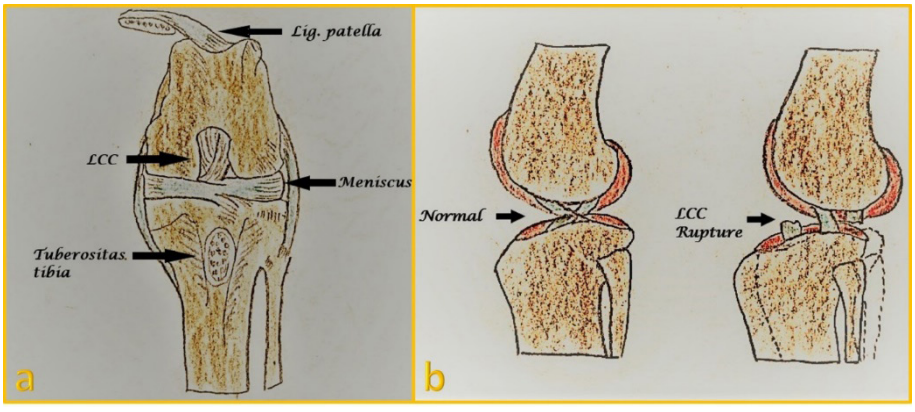

Figure 1: a) Anterior view of the cranial cruciate ligament, b) Normal and ruptured view of the cranial cruciate ligament.

3. General Information About Cranial Cruciate Ligament

Cranial cruciate ligament rupture is an issue that veterinary orthopedists have focused on for more than 40 years. Its pathogenesis is still not fully understood, but it is known to be multifactorial. (11,12) Mechanical, biological, hereditary, degenerative and immunological factors have been suggested. (12) 8% - 31% of cranial cruciate ligament ruptures in dogs are bilateral, and 18% - 61% are unilateral. (13)

Complete or partial rupture of the cranial cruciate ligament is one of the most common lesions that cause pain (14), muscle atrophy (15), hind limb lameness, and osteoarthritis. (1,5,14-22) While complete rupture of the cranial cruciate ligament causes severe lameness, partial ruptures cause inflammatory and pathological changes such as synovitis, osteoarthritis, meniscus damage and deterioration of the biomechanics of the knee joint. (5,19,23) Although it can be seen in all races, older and large races are predisposed. (24,25) Cranial cruciate ligament ruptures are much less common in cats compared to dogs (26-28).

4. Physiopathology of Cranial Cruciate Ligament Rupture

Cruciate ligament injuries have been associated with excessive force applied to the joint or sudden flexion of the knee between 20°-50°. (1) The cause of cranial cruciate ligament ruptures are usually due to degenerative changes in the cranial cruciate ligament (22,25,29), and ruptures may occur during normal daily activities without any trauma. (22,29) Physical examination, radiography (Figure 2), magnetic resonance imaging, arthroscopy and arthrotomy are commonly used in the diagnosis of cranial cruciate ligament ruptures. (24)

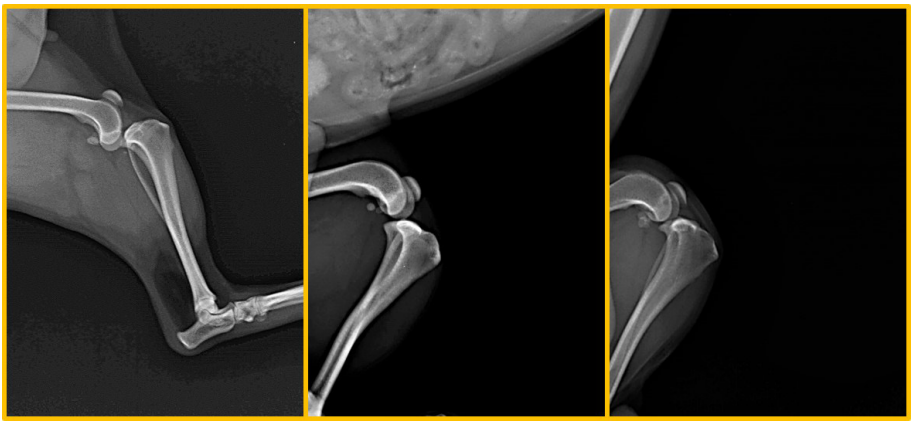

Figure 2: Mediolateral radiography images taken in tibial compression in cases with cranial cruciate ligament rupture.

5. Techniques Used in Cranial Cruciate Ligament Rupture

Although many surgical methods have been described in the repair of cranial cruciate ligament damage or rupture (1,11,16,17,22,24,30) none of these techniques has been universally accepted. (31) However; these techniques can be classified as intra-articular, extracapsular and osteotomy techniques. (4,32,33) Intraarticular techniques are Dickinson and Nunamaker Technique (Modified Fascia Lata Technique), Dueland Technique (Modified Patellar Tendon Technique) (1), Over-The-Top Technique (Arnoczky Technique) (1,6,32), Gortex Ligament Technique, Jones Technique, ligamentum cruciatum craniale (LCC) transfer. (6) Extracapsular techniques are Pearson Technique (Capsular Imbrication Technique), Hohn Technique (Postlateral Capsuloraphy) (1), DeAngelis Technique (Lateral Retinacular Imbrication Technique) (Figure 3a), Flo Technique (Modified Retinacular Imbrication Technique, Lateral Imbrication Technique) Olmstead Technique, Brown Technique and Fishing Line Technique (1,6), Three-in One Technique, Fibula Head Transposition, Tightrope Anterior Cruciate Ligament Technique. (32) Osteotomy techniques are Cranial Tibial Wedge Osteotomy (CTWO), Tuberositas Tibia Advancement Transport (TTA) (Figure 3b), Tibial Plateau Leveling Osteotomy (TPLO) (Figure 3c), Triple Tibial Osteotomy (TTO), (33), Combination of Tibial Plateau Correction Osteotomy and Cranial Closed Wedge Osteotomy, Proximal Tibial Intra-articular Ostectomy (PTIO), Chevron Wedge Tibial Osteotomy and Modified Maquet Technique. (32) The lateral extracapsular suture is still the most widely used surgical technique in small dogs. (11) In large and large breeds, the techniques of changing the level of the tibial plateau and moving the tuberositas tibia advancement have better results compared to other techniques. (34)

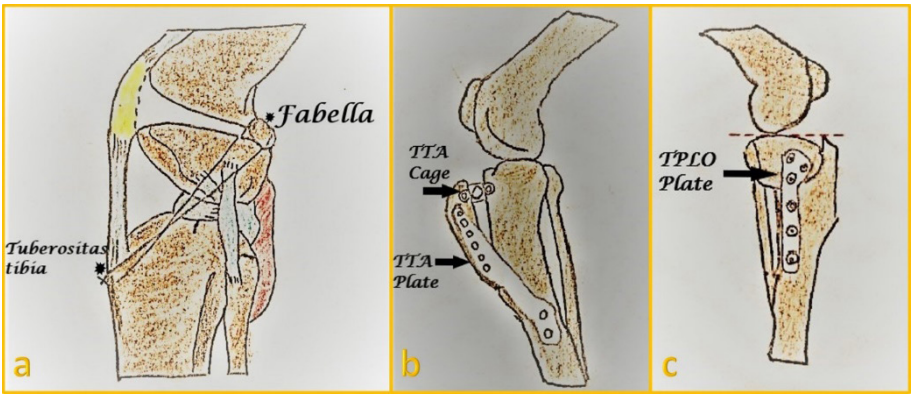

Figure 3: Schematic view of some techniques used in the treatment of cranial cruciate ligament rupture a: Fishing-line Technique, b: TTA Technique, c: TPLO Technique.

6. Complications

The complication rate in the classical TTA procedure is between 19% and 59%, and these complications are; meniscus tear, infection, medial luxation of the patella, chronic poor performance, implant failure, and tibia fracture. (12) In the TTA rapid technique, the minor complication rate is 30% and the major complication rate is 4%. (22) Complications arising from implant and tibial crest fracture in TTA technique may be related to the implant, tibial crest, or both. (35) Tuberositas tibia fractures are seen in 1% - 4.3% of patients. (12) Fixation of these fractures may be difficult due to the insufficiency of the existing bone tissue. In this case, pins, tension band, transarticular external fixators and dynamic compression plate (DCP) can be applied successfully. (35)

7. Treatment of Cranial Cruciate Ligament Rupture

Extracapsular stabilization methods are widely used in surgical treatment. The main factors to consider when deciding on the treatment to be applied are the patient's size and activity level. (14) Controlled activity, weight control, analgesic and anti-inflammatory drugs are recommended for the medical treatment of cranial cruciate ligament ruptures. (25,30) In addition, a patient weighing less than 15 kg increases the prognosis positively. (4,14,25,30)

7.1. Classic TTA and TTA-Rapid Technique

Classic TTA technique; In humans, it was first described in 1976 as the Maquet procedure to reduce patellofemoral joint contact and relieve patellofemoral pain. (21,36,37) Advacement of tuberositas tibia (TTA) was first introduced by Montavon and Tepic (2002) as a less invasive method in veterinary surgery as an alternative to TPLO. (36,38) The importance of the biomechanics of the knee joint in the repair of the cranial cruciate ligament in dogs is emphasized. Biomechanical studies show that the angle between the tibial plateau and the patellar ligament is responsible for the generation of tibiofemoral shear forces. (39) Techniques introduced by Sloccum; It is aimed at reducing cranial tibial thrust (cranial tibial thrust) by changing the geometry of the knee joint instead of focusing on repairing the torn ligament. The first attempts with the development of the tibial wedge osteotomy (TWO) were made in the early 1980s, and a decade later, the tibial plateau leveling osteotomy (TPLO) was developed by Sloccum. (22,40) New techniques aim to neutralize the femorotibial rupture force that causes cranial cruciate ligament ruptures. (21,41) In order to eliminate the rupture force, it is necessary to change the level of the tibial plateau or to

move the tuberositas tibia advancement. (2) The total force within the knee joint is approximately parallel to the ligamentum patellae, that is, to the functional axis of the tibia. (12) The angle between the ligamentum patellae and the tibial plateau is called the patellar tendon angle (PTA). (21,36,42,43) Normally, the angle of the knee joint is approximately 135°, and the patellar tendon angle is approximately 105°. (41,44) When this angle is 90°, the rupture forces are neutralized, the load on the cruciate ligaments is reduced to zero and the rupture forces are eliminated (Figure 4). (20,21,36,42-46) According to some studies, the flexion of the knee joint changes the PTA, and if the flexion angle increases, the PTA decreases, which causes less distance to be advanced in preoperative planning. (21)

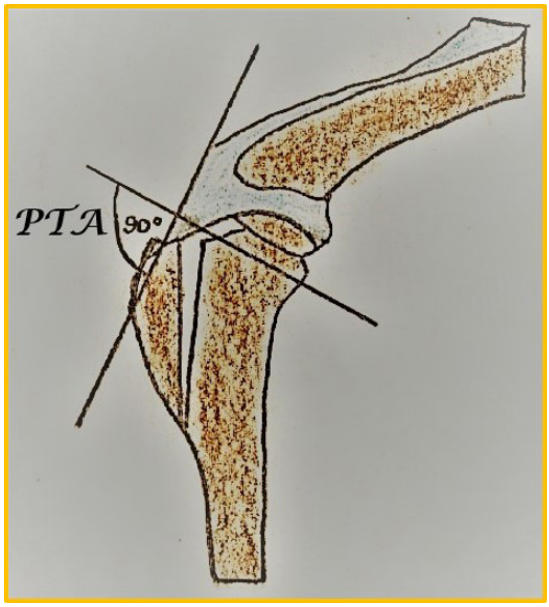

Figure 4: Schematic view of the patellar tendon angle (PTA) of 90° with the TTA technique.

TTA has been defined as a stabilization technique used in cranial cruciate ligament damage or rupture in the knee joint. (46,47) This procedure involves a longitudinal osteotomy of the tuberositas tibia. While full osteotomy is applied to the tibial crest in the Classic TTA technique, partial osteotomy is performed with a Maquet hole in the TTA-Rapid Technique. (22) During preoperative planning for TTA, a mediolateral radiograph is taken when the affected knee joint is in full extension position, and the size of the cage to be used is determined with a

transparent guide after the patellar tendon angle is determined (Figure 5). (21) It is fixed to the tibia by placing the appropriate "cage" on the osteotomy line. Then, autogenic or allogeneic bone grafts can be placed on the osteotomy line. (44) Recently, more allografts have been preferred in humans due to complications such as prolonged surgery time, fractures, bleeding and limited graft collection, but there is not much information about the complications resulting from the use of autografts in veterinary surgery. (47) Other reported grafts are xenograft, tricalsium phosphate and nanohydroxyapatite. (46)

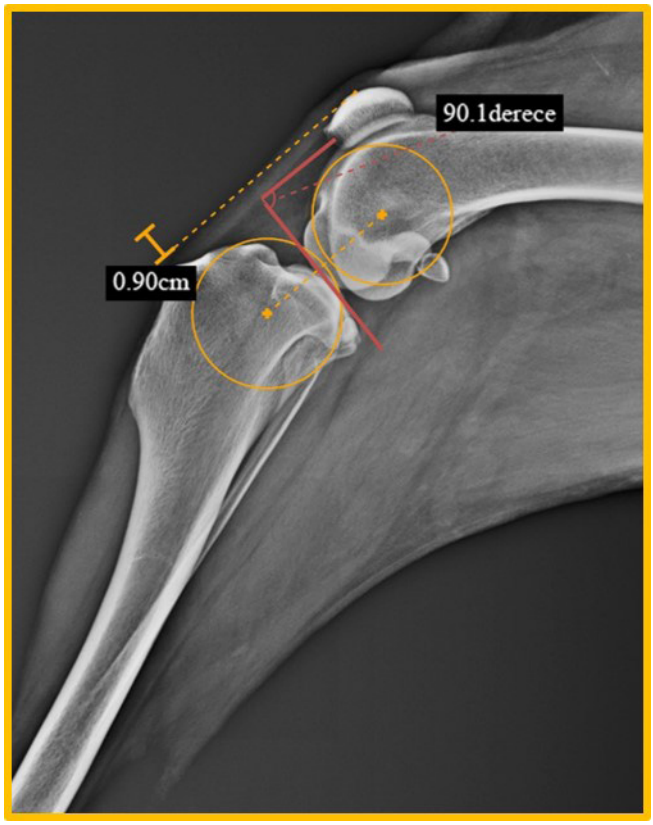

Figure 5: Drawing of TTA technique (yellow) and patellar tendon angle (red) on radiograph.

8. Advantages of TTA

TTA has many advantages over other repair techniques. Since the frontal plane osteotomy of tuberositas tibia is less invasive, it has less risk of changing the angular and rotational structure compared to other techniques. (45) Short

operation time (6,48), no need for post-operative bandage, rapid healing of the osteotomy line, protection of meniscus structures (6), less invasiveness, rapid return to normal health and it has advantages such as fewer complications. (6,45) In cases with ruptured cranial cruciate ligament, there is usually meniscus damage as the knee joint is unstable. In a study, it was reported that meniscal damage ranged from 33% to 77%, and late meniscal damage formed after surgery ranged from 3% to 17%. (49) Muscle atrophy is common following cranial cruciate ligament ruptures in dogs and may progress further after surgical intervention. (36)

9. Operative approach in TTA Technique

The incision line starts from the medial of the leg at the level of the medial meniscus and continues to the distal of the forearm of the saphenous vein. (6) The proximal tibia is then prepared for osteotomy. In the classical TTA Technique, TTA plate and cage are used to move the tuberositas tibia advancement. (36,38) In the TTA-Rapid technique, only a cage is used to move the tuberositas tibia advancement. (22) In the classical TTA Technique, the tibial crest is completely released, while in the TTA Rapid Technique, the osteotomy line is combined with a maquet hole created distal to the tibial crest. The operation line is closed with a suitable absorbable (polyglycolic acid) surgical suture material. Post-operative antibiotics and wound care are given for 1 week.

10. Clinical Evaluation Scales

Table 1: Lameness Evaluation Scale (50)

LAMENESS	
Grade	Evaluation
0	Free walking, equal load on all 4 legs
1	Appears to have reduced weight-bearing on the affected leg, but no lameness when walking
2	It does not burden the affected leg while standing, slightly lame when walking
3	Unwilling to stand or walk, has lameness when walking
4	It does not burden the affected leg while standing or walking, there is marked lameness

Table 2: Ilinios University Clinical Evaluation Scale (6)

KNEE JOINT MOBILITY/PALPATION	
Grade	Evaluation
1	No limitation of joint movement and no crepitation
1,5	No limitation of joint movement, palpable crepitation
2	Mild (10-20%) limitation of joint motion, no crepitation
3	Mild (10-20%) limitation of joint movement, crepitation
4	There is moderate limitation of joint movement and crepitation
5	There is severe limitation of joint movement (> 50%) and crepitation

Table 3: University of Colorado Clinical Pain Rating Scale (51)

PAIN	
Grade	Evaluation
1	No significant pain, minimal pain
2	There is mild pain
3	There is mild to moderate pain
4	There is moderate to severe pain

11. Conclusion

As a result, cranial cruciate ligament ruptures are frequently encountered in dogs. Many methods are used in cranial cruciate ligament ruptures. Techniques that change the mechanics of the knee joint have recently gained popularity. The success rate is quite high in surgeries performed by the advancement of tuberositas tibia.

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CHAPTER VII

PISCIRICKETTSIOSIS: A REVIEW ON ETIOLOGY, PATHOGENESIS AND TREATMENT

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1. Introduction

First identified in Chile in 1989, *Piscirickettsia salmonis* is the first obligate intracellular gram-negative bacterium identified in fish. Since then, it has been diagnosed in many regions of the world, including Turkey. Piscirickettsiosis is mainly seen in salmonid fishes but also in non-salmonid fish species such as Nile tilapia and sea bass. Following broodstock infection, fertilized eggs are also infected, and infected fry spread the causative agent through feces without showing symptoms of disease, making the infection of particular importance. The high mortality rates in outbreaks caused by the bacterium, which can cause disease in fish living in both freshwater and seawater environments, the development of resistance to antibiotics and the inability to demonstrate the effectiveness of the vaccines used make the disease economically important and cause serious economic losses in the aquaculture sector.

2. Etiology, Taxonomy and Genetic Diversity

The disease associated with *Piscirickettsia salmonis* was first observed in 1989 in a farm in Chile where coho salmon (*Oncorhynchus kisutch*) were farmed. (1) It was observed that clinical signs began to appear 6 to 12 weeks after the fish were transferred from freshwater to seawater. Researchers identified this outbreak, which had a cumulative mortality rate of 30-90%, as Coho Salmon Syndrome. (1) *Piscirickettsia salmonis* is a bacterium that has been difficult to classify since its first appearance. Initially called a rickettsia-like organism, *P. salmonis* was initially classified by phenotypic characterization as a member of the Alphaproteobacteria class in the Rickettsiales order in the family Rickettsiaceae and phylogenetically related to the genera Rickettsia, Wolbachia, Anaplasma and Ehrlichia. (2) Later, by closer analysis of 16S r-RNA it was classified as a new genus and species, *Piscirickettsia salmonis*, in the class Gammaproteobacteria in the order Thiotrichales under the family Rickettsiaceae, where it would be phylogenetically closer to the genera Francisella, Legionella and Coxiella. (3) Nowadays, it is included in the family Piscirickettsiaceae in the class Gammaproteobacteria as the species *Piscirickettsia salmonis*. (4,5) Unlike the genus Piscirickettsia, other members of this family are motile and morphologically rod, comma or spiral shaped bacteria. (6)

LF89 (ATCC VR-1361), the field strain of *Piscirickettsia salmonis*, was the first Gram-negative intracellular bacterium isolated from fish. (1) The bacterium, which does not grow on general bacterial media, can be grown in vitro on a specific cell line (CHSE-214) in cell culture with a long incubation period. (7)

In addition to the field strain LF89, other strains have been reported from different geographies. ATL-491 was isolated from Atlantic salmon in Canada in 1992 (8), NOR-92 from Atlantic salmon in Norway in 1992, SLGO-94 from rainbow trout (*Oncorhynchus mykiss*) in Chile in 1994 and CI-95 from coho salmon in Canada in 1995. (9) The strain of *P. salmonis* isolated from fish with severe encephalitis in European sea bass (*Dicentrarchus labrax*) in the Mediterranean Sea was associated with strain LF-89. (10)

In a study analyzing 500 field isolates of *P. salmonis*, it was shown that 50% of the samples were phylogenetically related to EM-90 or LF-89 isolates, and in addition, genetic groups were found to be widely distributed and responsible for outbreaks of Piscirickettsiosis in salmon farms. (11)

Different methods need to be applied to reveal significant differences in genomic and phenotypic characteristics between *P. salmonis* genogroups.

In a study that brought a different perspective to genotyping studies, several bacterial genes encoding molecular functions related to antioxidant activity, transcriptional factors, translation regulation and genogroup-specific virulence factors were discovered by comparative analysis of nuclear genomes. (12)

There is a genetically distinct *P. salmonis* variant obtained from *P. salmonis* infected fish by propagation in the CHSE-214 cell line. This variant was characterized by analyzing the sequences of ITS (Internal Transcribed Spacer Region) between the genes encoding 16S r-DNA and 23S r-DNA. The ITS region of this variant is different from the LF-89 strain and requires a different PCR primer sequence for amplification. This variant, which is not a new strain but an infective variant of the reference strain, is smaller than normal bacterial size (>0.2 μm) and can be recognized by standard antibodies against the bacterium. The ability of this variant of the bacterium to survive in seawater is the main basis on which it is held responsible for horizontal transmission in seawater. (13)

Piscirickettsia salmonis has two ITS regions, ITS A and ITS B. ITS sequence analysis of 11 *P. salmonis* isolated from different salmon species and geographical regions in Chile showed the presence of two distinct groups. ITSs with higher and lower electrophoretic mobility were detected, including isolates LF-89 and EM-90, respectively. This suggested that more than one r-RNA operon may be present in the bacterium. (14,15)

P. salmonis is also similar to Francisella spp. in terms of genetic characterization. (16) This situation necessitates the utilization of genes that are not present in the genus Francisella in the differential diagnosis and probably enable *P. salmonis* to survive in different ecological environments. (17)

3. Morphology and Phenotypic Characteristics of Bacteria

Fryer observed round cell clusters in the CHSE-214 (Chinook Salmon Embryo-214) line after 10 days of cultivation at 15 °C with a medium without antibiotics. (3) *P. salmonis* forms typical biofilm-like large cell clusters under stress conditions. When treated with cellulase, this matrix disappeared and the presence of polysaccharide structure in the biofilm layers was observed. They suggest that this structure may be a strategy for bacterial survival and persistence under stress conditions in the marine environment. (18)

Piscirickettsia salmonis is a gram-negative bacteria which is aerobic, usually non-motile, aerobic bacterium that has also been found to have a microscopically non-encapsulated, pleomorphic, usually coccoid, paired or

ring-shaped morphology of about 0.5-1.5 μm in diameter in Giemsa stains of tissues of infected cells. (2,3).

The bacterium grows in membrane-bound cytoplasmic vacuoles in tissue culture cells or host cells. The bacterium has unique nutritional requirements for growth in cell culture, which must be met with sensitivity for successful growth. (2,3)

Piscirickettsia salmonis has two surface membranes, an outer wavy membrane and an inner cytoplasmic membrane. The bacterium also has ribosomes near the plasma membrane and fibrillar DNA and electrodense spherical structures in the central region. (2)

It has also been shown that the strains show different phenotypic characteristics when *Piscirickettsia salmonis* is incubated in different culture media. While EM-90-like strains of the bacterium showed a phenotypically mucoid character in the culture medium when incubated at 22°C, no such phenotypic feature was observed in LF-89-like strains. (11,12)

4. Host Specificity of the Bacterium

The main affinity hosts of *P. salmonis* are salmonid fish. It can cause disease in freshwater fish as well as saltwater fish. Piscirickettsiosis has been reported in Chile in coho salmon (*Oncorhynchus kisutch*), rainbow trout, cherry salmon, *Oncorhynchus masou* (Walbaum) and Atlantic salmon. (1,19-21) The disease has caused chronic losses among chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) and pink salmon and *Oncorhynchus gorbuscha* (Walbaum) in British Columbia, western Canada. (22) In addition, Pisrickettsiosis has also been detected in Atlantic salmon in Ireland, Scotland and Norway. (23-25)

Piscirickettsia salmonis infection has also been observed in non-salmonid fish species in different parts of the world. In Taiwan, Nile Tilapia (*Oreochromis niloticus* L.) and single-spot groupers (*Epinephelus melanostigma*) were found to be susceptible to the disease agent. (26,27) In studies conducted in the United States, it was proved that blue-eyed plecostomus (*Panaque suttoni*) and white seabass (*Atractoscion nobilis*) were susceptible to the disease. (28,29) Studies have shown that *P. salmonis* is the natural infective agent of white perch, a commercially important fish in the northern hemisphere. This is a warning of the potential spread of the disease to other species. (29) The first outbreak was reported in juvenile European sea bass (*Dicentrarchus labrax*) showing abnormal swimming behavior off the southern Mediterranean coast of France.

(30) In Greece, Turkey and Croatia, *Piscirickettsia salmonis* has also been isolated from European sea bass and disease has been reported. (31-34)

5. Effective Environmental Conditions for Infection

Water salinity and temperature are important water parameters that affect the survival, pathogenicity and transmission potential of *P. salmonis* bacteria outside the host. While *P. salmonis* can survive for an extended period in seawater, it is rapidly inactivated in fresh water. In an experimental study showing the effect of water temperatures on the survival of the pathogen, *P. salmonis* survived in seawater for at least 21 days at 5-10 °C, 14 days at 15 °C and 7 days at 20 °C, but not at temperatures above 25 °C. The rarity of outbreaks in freshwater may suggest that the instability of the bacterium in this environment may be the cause and that seawater may be the source of the disease. (35)

The highest incidence of outbreaks is observed in the fall and spring seasons when the water temperature is between 9-16 °C. In the outbreak in Greece between 1995 and 1999, the mortality rate reached 30% in hatcheries and 80% in cages during the colder months of December and March when the temperature was 10-16 °C. (36)

6. Bacteria Transmission Routes, Vectors and Reservoirs

The pathogen can enter the host orally, through the gills or through the skin. Although *P. salmonis* is able to penetrate intact skin and gills, the risk of infection is increased by the portantries that open following damage to these organs. The bacteria are excreted from live fish in bile, feces and urine. Fecal excretion provides another route of transmission for infection by coprophagy. (37-39) In experimental studies, intraperitoneally and gill-infected fish showed higher mortality than orally infected fish. (40) The incubation period for Piscirickettsiosis under natural conditions is thought to be 10-14 days. (41)

The bacterium can replicate in insect and frog derived cell lines and may have the potential to survive in invertebrates and non-fish poikilotherms. (42)

Piscirickettsia salmonis has been detected in the gonads, genital fluids and testes of naturally infected salmon. (43-45) In intraperitoneal bacterial inoculation, moderate levels of *P. salmonis* were detected in 10% of fertilized eggs of male and female rainbow trout, female genital epithelial fluid and seminal fluid. (46) In another study of intraperitoneally infected with *P. salmonis*, all fertilized eggs obtained from male and female broodstock fish were found to be

infected, but the eggs remained viable for hatching. Infected fry did not show signs of disease but spread the agent through feces. (46) Piscericketsiosis is rare in the freshwater life cycle of salmon. Therefore, it is thought that vertical transmission may not be common for *P. salmonis.* (48)

Ceratothoa gaudichaudii, a parasitic isopod commonly found in salmonids, was identified as a vector of *P. salmonis* by indirect fluorescent antibody testing. (49)

7. Piscirickettsiosis

7.1. Bacterial Pathogenesis

The pathogenesis, pathogenic mechanisms and immune evasion mechanisms of *P. salmonis* are poorly understood. (50,51) The virulence factors of *P. salmonis* are poorly known. (41) *P. salmonis* is known to secrete extracellular products (ECPs) and these are known to exert cytotoxic effects in vitro. These ECPs mediate tissue damage in trout tissue in vivo. Due to the peptidic nature of *P. salmonis* ECPs, their cytotoxic effect is inhibited when treated with proteinase K in vitro. Therefore, they can be categorized as exotoxins. Many of these ECPs are thermolabile exotoxins possibly playing a role in the pathogenesis of Piscirickettsiosis. (52)

The functional presence of four genes (dotB, dotA, icmK and icmE) homologous to Dot/Icm type IV secretion systems (SSTIVs) has been identified for *P. salmonis*. The dot/Icm system is the main virulence mechanism of the intracellular pathogens *Legionella pneumophila* and *Coxiella burnetii* and is responsible for their intracellular survival and proliferation. It is thought that this may also be the case for *P. salmonis*. After being phagocytized by macrophages, *P. salmonis* survives and proliferates in the vacuole by preventing lysosomal fusion. (53) The interaction of the Dot/Icm secretion system within the host cell and the bacterium appears to be responsible. (50,54) *P. salmonis*-infected macrophages were observed to have fewer lysosomes compared to those incubated with inactivated *P. salmonis*, and infected macrophages were reported to have less proteolytic activity. (55) It has also been suggested that *P. salmonis* induces an anti-inflammatory environment when it infects macrophages by manipulating the host cytokine profile to promote a favorable environment for its survival and replication in macrophage-like cells. (56)

Rojas et al. reported in 2010 that *P. salmonis* induced apoptosis in macrophages of salmonids infected in vitro at early, middle and late stages

of infection. However, the exact relationship of apoptotic cell death with the pathogenesis of Piscericketsiosis is not clear. Therefore, researchers speculate that, in addition to other strategies to evade host immune responses, such as inhibiting phagolysosomal vacuoles, induction of macrophage apoptosis may represent a mechanism by which *P. salmonis* evades the host immune response and establishes efficient infections. It has also been reported that *P. salmonis* induces apoptosis in salmon phagocytic cells but not in epithelial cells. (57)

P. salmonis has been shown to use a clathrin-dependent endocytosis pathway as a mechanism to enter fish macrophages. (58) Once inside the cell, *P. salmonis* gradually loses its association with clathrin and reorganizes the actin cytoskeleton to form actin-containing vesicles that support bacterial replication. In the later stages of infection, the affected cell also induces novoactin synthesis as a defense strategy to restructure the cytoskeleton; however, the bacterium uses the newly synthesized actin molecules to form macrovesicles oriented to the outer membrane and facilitate the release of newly formed bacteria. (58)

7.2. Host Immunity

P. salmonis activates innate immunity, inducing inflammatory reactions in the anterior kidney and an interferon-mediated response in the liver. In the adaptive immune response in infected fish, down-regulation of genes involved in the G protein signaling pathway and apoptotic process is thought to reflect mechanisms used by *P. salmonis* to survive, reproduce and evade host defenses. (59,60) Thus, the infective strategy of *P. salmonis* for intracellular survival (54) is evasion of host immunity (50,59,60) and the activation of the host's immunity for a short period of time. However, studies contributing to a better understanding of the immunological activity and bacterial factors involved in the disease are still limited. (41)

7.3. Clinical and Necropsy Findings

Fish severely affected by *P. salmonis* show hyperpigmentation anorexia, lethargy and irregular swimming near the surface, whereas inspection of those less affected shows no abnormalities. (8,25,44,45) The most common external lesions include pale gills as a result of severe anemia, volumetric increase in the abdomen, and petechial and ecchymotic hemorrhages at the base of the fins. (41) The most common visceral findings include ascites, splenomegaly and reno-splenomegaly. (41,43,45) Systemic infection also affects other organs such as the liver, ovary and brain. (2,61) The clinical signs and salient features of salmon

ricketsial syndrome vary depending on whether there is an acute outbreak or whether the affected fish are chronically infected. In peracute mortalities, extensive internal hemorrhages may be observed without any external changes. In chronic cases, agonic fish are dark and lethargic, with pale gills and often small nodules on the fins that can ulcerate. Such fish have swollen kidneys, enlarged spleen and mottled liver. This is usually accompanied by petechial hemorrhage along the muscle and pseudomembranous exudate covering the pericardium and visceral peritoneum and internal organs. (44,45)

Between 1995 and 1999, 2000 sea bass larvae (0.2-1.5g) from two production and supply farms and 450 juvenile sea bass (weight 2-15g) from five cage farms in Greece were examined. Clinical signs in the hatcheries were lethargy, loss of appetite and discoloration, while sea bass transferred from hatcheries with the disease to sea cages were found to show irregular swimming, loss of orientation, lethargy and abnormal behavior after twenty days. Mortality reached 30% in hatcheries and 80% in cages during the colder months of the year (10-16 oC) (December-March). (36)

In Croatia, between 2013 and 2015, starting in December when sea temperatures dropped below 16°C, outbreaks were observed in cage farms of European sea bass in Croatia, with cumulative mortality of 20-30%, and continuing until sea temperatures rose in May. (34) In both outbreaks, affected fish died with the same erratic swimming and turning behavior and lack of interest in food. At necropsy, corneal opacity, pallor of the liver, enlarged gallbladder with bile accumulation, generally no contents in the gastrointestinal tract, and widespread congestion of the meninges were observed. Based on the initial findings, it was thought by the researchers that this outbreak might be associated with viral necrosis of the nervous system (VNN) and real time PCR was negative for this infectious agent. (34)

7.4. Histopathology of Piscirickettsiosis

Salmon rickettsial septicemia in Atlantic salmon is often characterized by multifocal, necrotic areas of hepatic parenchyma. Histopathologically, the formation of pyogranulomatous/granulomatous lesions in the liver, spleen and kidney is typical. In the early stage of infection, young granulomas are typically necrotic in the center, surrounded by macrophages and numerous polymorphnuclear leukocytes. Older granulomas consist of a central necrosis surrounded by strong connective tissue and fewer inflammatory cells. In addition, perivascular infiltration of macrophages is a typical finding. (25,62,63)

In 2005, Arkush et al. recorded histopathologic findings in all visceral organs, especially liver and spleen in white sea bass. The lesions in the liver, defined as the primary target organ for the bacteria, were emphasized as sometimes granulomas in foreign body formation, macrophage clusters, capsular inflammation, focal foci of necrosis. In addition, diffuse hepatocellular glycogen depletion, single-cell necrosis, spongiosis hepatis and hepatocellular regeneration were observed. Granulomas were also found in the mesentery and serosa. In the spleen, macrophage clusters were observed mostly in the sinusoids. It was emphasized that mononuclear cells, especially macrophages, accumulated to occlude sinusoids and large vessels. Among renal lesions, interstitial granulomas, focal/diffuse macrophage infiltration, protein droplets in the proximal tubular epithelium were described. While granulomatous inflammation was noted in the gills, it was reported that few lymphocytes and many macrophages infiltrated the filaments and lamellae. Foci of necrosis with severe granulomatous inflammation, single-cell necrosis, small hemorrhages and prominent eosinophilic, intracytoplasmic bacilli in macrophages and epithelial cells infected with *Piscirickettsia salmonis* were observed. (29)

In a 2017 study by Rozas-Serri et al. comparing the pathogenesis of LF-89-like and EM-90-like *Piscirickettsia salmonis* isolates in the early and late stages of the disease in Atlantic salmon, findings in the early stage of the disease showed a slight and gradual increase in the number of melano-macrophage centers in the periportal space of the liver on day 14 of infection in both isolate trials. This was more pronounced in the EM-90 isolate experiment than in the LF-89 isolate experiment. On the 21st day of infection, liver sinusoids were enlarged by erythrocytes and leukocytes and vacuolar degeneration was observed in hepatocytes in both groups. After the 35th day of infection, degenerative changes in the liver shifted to focal necrosis and accompanied by histiocytic infiltration in the EM-90 isolate experiment, while these changes were observed on the 42nd day of infection in the LF-89 isolate experiment. Again on day 42, both isolate experiments showed moderate multifocal conglomerated granulomas with the presence of *P. salmonis* in hepatocytes and macrophage-like cells. In the kidneys, a slight increase in the number of melano-macrophage centers was noted on day 14 of infection in both isolate experiments, while focal proliferative glomerulonephritis, polymorphonuclear cell infiltration and hematopoietic tissue hyperplasia were observed on days 21 and 28. On the 42nd day of infection, tubulonephrosis and combined conglomerated multifocal granulomas in the kidney were observed in both isolate trials. It was noted that cardiac

lesions that started with cardiomyocyte degeneration turned into cardiomyocyte necrosis on the 35th day of infection and mononuclear cell infiltration was observed in the epicardium and myocardium in both groups. Perivascular polymorphonuclear infiltration in the parenchyma and meninges, hemorrhage in the meninges and thrombosis in the meningial vessels with increasing severity with the progression of the pathogenesis process were listed as the lesions seen in the brain regardless of group. Mild epithelial cell degeneration in the epidermis and mild necrosis of subdermal muscles were listed as skin lesions; mild polymorphonuclear infiltration on day 35 of infection was recorded in addition to these lesions only in the EM-90 isolate trial. In the intestines, only in the EM-90 isolate trial, congestion in the lamina propria and mononuclear cell infiltration in the intestinal submucosa were emphasized. In the gills of infected fish in the EM-90 isolate trial, multifocal apical epithelial hyperplasia and fusion of the secondary gill lamellae and mild necrosis of isolated epithelial cells in the secondary gill lamellae were observed, while no significant microscopic changes were reported in the gills of fish infected with LF-89 isolate. Increased numbers of melano-macrophage centers, severe hyperplasia of hematopoietic tissue and severe diffuse proliferative glomerulonephritis were observed in the renal interstitium. (64)

Specifically, in sea bass larvae (0.2 -1.5g) and juvenile sea bass (2-15g) examined in Greece (Dicentrarchus labrax), the subcutaneous and skeletal systems of the affected individual showed necrotizing, mixed leukocytic dermatitis, osteochondritis and diffuse gill inflammation, often dominated by granulocytes and rickettsia-laden histiocytes. Similar integumental inflammation has been detected along the cranial sensory canals and nostrils. In the central nervous system, inflammatory reactions progressed to the optic and olfactory sensory nerves and retina in the form of perineuritis and necrotizing congestive meningoencephalitis. Evidence for the transfer of infective agents across the blood-brain barrier was confirmed by the presence of immunohistochemically-positive rickettsiae in the capillary endothelium and immunopositivity in histiocytes in inflamed areas of the optic tectum and cerebellum. In the most severe cases, the infection spread to the stacoacoustic canal system and the ependymal surface of the ventricles with infiltration of rickettsiae-laden histiocytes. Visceral organs were equally affected, with moderate to severe necrotizing histiocytic gastritis and hepatitis. This rickettsial agent was also easily identified immunohistochemically in the gastric submucosa and shed mucosal epithelial cells. (36)

In Turkey, the disease was first reported as a Rickettsia-like organism in a sea bass farm on the Black Sea coast in 2003. (33) In 3 different farms where European sea bass (Dicentrachus labrax) were farmed, mortality rates up to 30% were recorded and Vibrio anguillarium and Photobacterium damsela subsp. piscicida were isolated from the sick fish in microbiological analysis. Basophilic granules associated with Rickettsia-like organisms were observed in monocytes in blood frog and in vacuolated cells with enlarged cytoplasm mainly in kidney, spleen and liver in histopathological examinations. (33, 65) Immunohistochemically, the presence of *Piscirickettsia salmonis* was demonstrated using anti-*Piscirickettsia salmonis* polyclonal antibody as described by McCarthy et al.. (10)

In September 2010, light and electron microscopic studies conducted in a facility in the Aegean Sea where sea bass mortalities were observed in September 2010 when the water temperature was 25 ^{o}C, Rickettsia like organism related disease was diagnosed. (66) Histopathologically, central necrotic epithelioid granulomas were determined as in Tilapia, White sea bass and European sea bass. In tissue sections, the causative agent was found to be Gram negative and Ziehl Neelsen negative. In May-Grünwald-Giemsa stained blood froths, many monocytes were found to contain small, basophilic, pleomorphic, coccoid Rickettsia-like organisms in vacuoles. Transmission electron microscopic examination of blood samples showed basophilic pleomorphic coccoid cells within large vacuoles of phagocytic blood cells. (66)

Affected European sea bass in Croatia showed lesions in all regions of the brain, including the medulla oblongata. In the brain, severe chronic encephalitis with moderate chronic lymphohistiocytic meningitis was identified. Necrotic foci with inflammatory cell infiltration were seen especially in the cortex of the optic lobes. In some areas, it has been reported that cell infiltrations with basophilic cytoplasm, vacuolar and globular morphology, mostly composed of macrophages, were observed in 2-3 rows in some areas. (34) Moderate lymphohistiocytic epicarditis was observed in the heart and no histopathologic lesions were observed in other organs examined. Positive staining of bacterial colonies was observed in brain sections by fluorescence in situ hybridization using a probe specifically designed for *P. salmonis.* (34)

7.5. Diagnostic Methods of Piscirickettsiosis

For the diagnosis of Piscirickettsiosis, smears preferably prepared from kidney, liver and spleen tissues of clinically symptomatic fish are stained

with Gram, Giemsa and Methilen-blue stains to visualize the causative agents microscopically. (2,5) Fluorescent antibodies and enzymatic immunoassays (ELISA), primers designed to amplify specific sequences of the 16S r-RNA gene or PCR analysis using the ITS-1 sequence located between 16S r-RNA and 23S r-RNA can also be deployed. (67-70) In immunohistochemical examinations using rabbit anti-*Piscirickettsia salmonis* antiserum; macrophages and hepatocytes in inflamed and necrotic areas in the liver, endothelium and myocytes in the heart and macrophages in the meninges. (25) *P. salmonis* has been difficult to grow on solid media. Until a 2008 study, it was thought that it could only be grown in eukaryotic cell cultures because it is an intracellular bacterium. (41,71) *P. salmonis* can be grown in cell lines and bacterial cultures. (2,67,71-73) The fact that *P. salmonis* induces apoptosis by actively targeting phagocytic cells provides an opportunity to study infectivity in macrophage-like cell lines. (74)

In an experiment using LF-89, ATL-4-91 and NOR-92 strains of *P. salmonis*, the attempt of growing the bacteria on blood agar was made. A new medium was designed by adding 3% fetal bovine serum (FBS), 0.1% cysteine and 1% glucose to 5% sheep blood agar. After cultivation on this medium (incubation at 16 °C for 6 days), it was reported that 0.1 mm, circular, convex and white colonies were reproduced. (71) Due to the risk of contamination in agars prepared with the addition of blood, two blood-free media have been proposed for cultivation, namely tryptone soy agar with ferric nitrate (Austral-TSFe) and tryptone soy agar with hemoglobin (Austral-TSHem). Austral-TSHem medium is reported to be the most suitable medium giving the highest number of cells per petri dish. (75) SRS-BA, Austral-TSHem, CHAB, CHAB containing 0.2 mM Fe, blood agar (BA), 2% NaCl BA, Marine agar (MA) and glucose agar with tryptone-yeast extract salts (FLPA) were incubated for 14 days at different temperatures and the bacterial colonies grown were compared. SRS-BA was found to be the agar on which 20 isolates of the bacteria grew best. In addition, 5 isolates grew only on SRS-BA. (76)

A marine-based broth supplemented with L-cysteine called Austral-SRS broth was designed to facilitate the growth of *P. salmonis* strains. The strains cultivated with this broth reached an optical density of approximately 1.8 when absorbance at 600 nm was measured after 6 days of incubation at 18°C. Genetic and serological studies were made suitable with DNA obtained from *P. salmonis* produced in this liquid medium, purification of lipopolysaccharide (LPS) and whole membrane protein. The cytopathic effect test showed that *P. salmonis*

produced in Austral-SRS broth retained virulence properties and induced apoptosis. For these reasons, it was recommended as an effective medium for the development of a low-cost vaccines. (73)

7.6. Disease Control, Treatment and Prevention

In today's aquaculture, many types of vaccines and antibiotics can be used in the treatment of bacterial diseases. In a study in which streptomycin, gentamicin and tetracycline were tested, it was observed that the bacteria were susceptible to these antibiotics but resistant to penicillin and oxolinic acid, and tetracycline was recommended for treatment. (2,77) Florfenicol and oxytetracycline, which is an antibiotic with bacteriostatic effect and frequently used in salmon farming, were not effective in the control of Piscericketsiosis. (77,78) According to data from the Chilean National Fisheries and Aquaculture Service (SERNAPESCA), 98.3% of the total antibiotics administered for the control of Salmon Rickersial Septicemia associated with Piscericketsiosis in seawater are mainly comprised of oxytetracycline and flufenicol. The aquaculture industry is constantly threatened by infectious diseases. Although antibiotics and other chemotherapeutics have proven to be beneficial, their environmental and economic handicaps must be considered. Therefore, immunoprophylaxis needs to be developed and implemented for disease prevention and control. Vaccination is one of the most important prophylactic tools for disease control in modern industrial aquaculture. (79,80) Commercial vaccines against *P. salmonis* infection that can be administered intraperitoneally or orally are available, but they show variable results. Vaccinated fish become infected with *P. salmonis* towards the end of the production cycle and die close to harvest, causing severe economic losses. In Chile, injection vaccines are used as a booster when antibody titers of oral vaccines drop near harvest. Oral vaccination can protect fish against *P. salmonis* when administered as primary vaccination or as a booster for an injected vaccine. (41,81,82) In general, vaccinated fish showed lower cumulative mortality rates than unvaccinated fish until winter, but vaccine efficacy decreased significantly in early spring and mortality increased in both vaccinated and unvaccinated fish. (83) All injectable vaccines are relatively effective in preventing the first outbreaks of Piscirickettsiosis that occur after the transfer of fish from freshwater to seawater, but after the initial outbreak threat, fish become susceptible to new and more aggressive outbreaks of Piscirickettsiosis as the specific immunity provided by the vaccine weakens. (81) These outbreaks usually affect large fish and occur 10-12 months after transfer, causing greater economic losses.

Subjecting these fish to an injectable revaccination program is not considered a viable method due to economic and practical difficulties. (41)

A live vaccine prepared from the avirulent *Arthrobacter davidanieli*, which has been used to prevent *Renibacterium salmoninarum* infection in salmon in Canada, Chile and the USA, has been shown to reduce mortality in experimental infection of coho salmon with *Piscirickettsia salmonis* and was reported to reduce mortality in coho salmon over a 10-month period after transfer to sea. (84)

Passive immunization with antibodies has attracted attention as an alternative approach to antibiotics due to its high specificity. Recently, chicken egg yolk immunoglobulin (Ig-Y) has attracted great interest as a potential method to prevent and control diseases. Ig-Y is a polyclonal antibody that can be easily obtained non-invasively from egg yolk and can recognize multiple epitopes. (85) In a study in which Ig-Y was obtained by immunizing chickens with *P. salmonis*, it was shown that anti *P. salmonis* Ig-Y inhibited the growth of the bacterium in liquid media and protected SHK-1 cells against infection in cell culture. When the same specific Ig-Ys were encapsulated and fed to Atlantic salmon, the antibody was absorbed from the intestinal mucosa and detected in serum. (86)

As in every disease, biosecurity measures are very important in the prevention and protection from Piscirickettsiosis. Cleaning and disinfection are the most important of these. Active chlorine dioxide, inactive chlorine dioxide, glutaraldehyde, hypochlorites, peracetic acid and peroxides have been tested and it has been shown that most compounds reduce the number of bacteria and peracetic acid and peroxides are more effective compared to other disinfectants. (87) It is suggested that the use of peracetic acid (1 min/10 ppm) would be the best choice for biosafety in a situation requiring the use of disinfectants against *P. salmonis*. (87)

8. Conclusion

P. salmonis affects certain fish species cultivated in salt water and causes mortality. The resistance of the disease to antimicrobials makes it difficult to control outbreaks and causes serious economic losses in the aquaculture sector with the amount of drugs used and fish lost.

In 2006, before the infectious salmon anemia (ISA) crisis in Chile, the Institute of Salmon Technology (INTESAL) estimated the direct economic losses

caused by Piscirickettsiosis during the growth phase in seawater to be about US$100 million. (88) These data represented about 25% of the total economic income from salmon exports in the same year. With the efficient reactivation of the Chilean salmon industry after the ISA crisis, the disease has re-emerged as the main health problem in the sector, thus raising again the issues associated with diagnosis, treatment, prophylaxis and control. (89)

The adaptability of piscicricketsiosis allows outbreaks to become increasingly insidious, resistant to treatments, and of increasing virulence under similar conditions. (83,91) Although the strategy for disease control in salmon relies on antimicrobials and vaccines, commercial *P. salmonis* vaccines have not demonstrated high efficacy. (83,90,91) Studies contributing to a better understanding of the immunologic activity and bacterial factors involved in the disease are still limited. (41) There is a need for studies to close this gap. Especially the fact that it is a pathogen seen in European sea bass makes all kinds of studies in this field crucial. Pathogenesis studies will pave the way for eliminating the necessary questions in the diagnostic and treatment approach in cases where the disease is seen in the aquaculture sector.

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