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EDITOR

ASSOC. PROF. DR. KEZBAN ŞAHNA

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Chapter 1

CELL TYPE IDENTIFICATION TECHNIQUES

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1. INTRODUCTION

1.1. Determination of Cell Identity and Techniques Applied for This Purpose

Current research and treatment approaches for cell identification primarily focus on methods and techniques that minimize human error, prioritize automation, and present results in the form of numerical data.

The main objective of these approaches is the identification and characterization of cells, and, when necessary, the isolation of either single cells or homogeneous populations consisting of a single cell type. Therefore, a marker capable of accurately defining each cell type is essential.

More than 220 different cell types have been identified in the human body. In traditional classification, parameters such as cell morphology, size, embryonic germ layer of differentiation, cell-specific structural components or secretory products, and basic functional features have generally been taken as the basis for establishing a form–function relationship.

In recent years, however, cell identification methods have been developed that rely on nuclear DNA (deoxyribonucleic acid) content, cell membrane surface molecules, or specific cytoplasmic molecules detected by immunofluorescence, enzyme-immunohistochemistry, or, particularly for DNA, fluorescent DNA-specific dyes. With advancements in computer science, electronics, and software technologies, both individual cells and cell populations composed of millions of identical cells can be rapidly identified and separated by automated systems within seconds. The most concrete example of this is the spermatozoon-sorting technology applied in human and veterinary medicine, which distinguishes X- and Y-chromosome-bearing sperm cells.

This section aims to provide an overview of the techniques and technologies utilized for cell typing and separation.

2. CELL DIFFERENTIATION

Cell differentiation refers to the structural, biochemical, functional, and metabolic changes that a cell undergoes during specialization for a particular function. This process begins with the formation of the zygote in multicellular organisms and continues until the generation of various cell and tissue types capable of performing specific functions is completed. In mature mammals, there are three principal cell groups: germ cells, somatic cells, and stem cells (Görmüş, 2011; Barry, 2018).

Germ cells are the founding cells of all sexually reproducing organisms and are responsible for the production of female and male gametes (ovum and spermatozoon), which unite through fertilization to form a new organism

(Wylie, 1999; Cinalli et al., 2008). Somatic cells contain diploid genetic material that includes the complete genetic information, and they specialize by differentiating according to the body's needs, performing functions appropriate to their type. Stem cells, on the other hand, are cells that play a role in the regeneration of mammalian tissues and have a high capacity for self-renewal (Öktem & Altay, 2009; Özer et al., 2021). Stem cells can differentiate into all mature cell types in the living body. From the embryonic stage, there are 5 different types of stem cells in the body. These are: embryonic inner cell mass (ICM) stem cell, amniotic epithelial stem cell, fetal stem cell, Umbilical cord epithelial stem cell, and Adult somatic stem cell (Charitos et al., 2021).

Although there are variations among researchers, the differentiation capacities of these cells are generally classified from highest to lowest as totipotent, pluripotent, multipotent, oligopotent, and unipotent (Abatay Sel, 2021). The zygote is the most important totipotent cell that has the potential to form all cell types in an organism, including extraembryonic placental cells (Şahin et al., 2005; Sağsöz & Ketani, 2008; Matur & Solmaz, 2011). It is known that each of the blastomeres forming the embryo at the eight-cell stage possesses totipotent capacity.

After the formation of the 3 basic embryonic layers consisting of ectoderm, mesoderm, and endoderm at the blastula stage, the cells of each layer can differentiate into 220 different cell types in the tissues and organs that develop from them. However, since they cannot transform into extraembryonic placental cells, they are called pluripotent cells.

Multipotent cells, on the other hand, have a more limited capacity; they can only form different cell types belonging to a specific tissue or cell group (Görmüş, 2011; Barry, 2018; Özer et al., 2021). The ability of hematopoietic stem cells to generate various cell lines such as erythrocytes, leukocytes, and thrombocytes is given as an example of this capacity (Lee & Hong, 2020).

Oligopotent cells are more limited in scope than multipotent cells and are characterized by the capacity to differentiate into only a few cell types. The ability of lymphoid progenitor cells to form T and B lymphocytes is included in this group.

Unipotent cells, on the other hand, have the capacity to differentiate into only a single cell type, yet they are considered stem cells due to their ability to maintain self-renewal. The ability of skeletal muscle satellite cells to differentiate only into myocytes is considered a specific example of this cell group (Yin & Rudnicki, 2013).

3. DETERMINATION OF CELL TYPES BY TRADITIONAL METHODS

It is well known that during embryonic development, each cell acquires the most suitable structure, shape, size, and metabolic characteristics for the function it will assume as a result of the differentiation process. Therefore, the cells that form tissues and organs have been typed and classified based on the form-function relationship (Table 1.) (Özer et al., 2021).

Table 1. *Basic tissues in the body and the traditional classification of cells in these tissues*

Epithelial tissues	**Covering Epithelium:** · **According to cell shape:** squamous, cuboidal, columnar (prismatic), and pyramidal. (In general, cell shapes include: oval/ovoid, spherical, squamous, cuboidal, columnar, pyramidal, cylindrical, stellate/ asteroid, fibrous, granular, polyhedral and polygonal.), · **According to the number of cell layers:** simple, pseudostratified, and stratified, · **According to both layering and cell shape:** **Simple epithelium:** simple squamous, simple cuboidal, simple columnar (prismatic) and simple pyramidal epithelium, **Stratified epithelium:** stratified squamous, stratified cuboidal, stratified columnar and pseudostratified (transitional) epithelium, **Pseudostratified epithelium:** pseudostratified columnar epithelium.
	Glandular Epithelium (Secretory Epithelium): · **According to the number of cells:** unicellular, multicellular, microscopic, and anatomical, · **According to the shape of the secretory unit:** **Simple glands:** tubular, alveolar, acinar, **Compound glands:** tubulo-alveolar. · **According to the nature of the secretion:** serous, mucous, or seromucous (mixed). · **According to the route of secretion:** exocrine and endocrine.
	Myoepithelium: These contractile cells, present in certain glandular tissues such as the mammary and salivary glands, surround and support secretory cells, thereby facilitating the expulsion of secretions.
	Neuroepithelium: Neuroepithelial cells are specialized cells responsible for detecting sensory stimuli, including hearing, smell, taste, as well as pressure, temperature, and cold.

Connective and support tissues	**Connective Tissue:** · **Embryonic connective tissues:** mesenchyme, mucous connective tissue (in adults: dental pulp and the comb of roosters). · **Adult connective tissues:** loose connective tissue, reticular connective tissue, dense connective tissue (with collagenous and elastic fibers, either regular or irregular), adipose tissue (white and brown adipose tissue). · **Cartilaginous Tissue:** hyaline, elastic, and fibrous cartilage. **Bone Tissue:** · **Fetal bone tissue** · **Adult bone tissue:** compact (cortical) bone and spongy (trabecular) bone. **Blood Tissue:** contains numerous distinct cell types. **Lymph Fluid:** contains white blood cells.
Muscle Tissues	**Striated muscle tissue:** includes skeletal and cardiac muscles. **Smooth (non-striated) muscle tissue:** composed of a single type of muscle cell.
Nervous Tissue	**Nerve Cells (Neurons):** consist of neurons with various shapes and functional roles. **Glial Cells:** include ependymal cells, astrocytes (protoplasmic and fibrous astrocytes), oligodendrocytes, microglial cells, and Schwann cells.

The four basic tissue types shown in Table 1. contain numerous different cells, which are traditionally classified based on their location, shape, and function. In this classification, parenchymal cells and stromal cell types are considered separately based on tissue types. Additionally, the primary embryonic layers from which cell types originate are taken into account in the classification. Table 2. presents the cell types defined according to this classification (Table 2.).

Table 2. *Cell types in the body that can be identified with a light microscope*

Cell Type	Location	Function	Additional Information
1. Myoepithel	Around the excretory ducts of glands	Assists in the expulsion of secretions.	Contains actin filaments and possesses contractile ability
2. Erythrocyte	Bone marrow, blood vessels	O_2 transport due to hemoglobin	Mature erythrocytes in mammals are non-nucleated, biconcave in shape, and acidophilic

3. Lymphocyte	Bone marrow, lymphatic tissues, blood and lymph vessels, intraepithelial and loose connective tissues	Significant roles in the immune responses with T, B, and NK subtypes	B lymphocytes mediate humoral immunity; T lymphocytes mediate cellular immunity
4. Monocyte	Bone marrow, blood, lymph, and other tissues	Phagocytosis	Motile cells that are precursors to macrophages.
5. Macrophage	Predominantly in connective tissues	Phagocytosis	It is a large cell transformed from monocytes, with an oval/bean-shaped nucleus and abundant cytoplasm.
6. Giant Cell	In tissues with chronic inflammation and certain specific disease agents, as well as in tumor tissues	Inflammatory response	Formed by cytoplasmic fusion of macrophages; includes Langhans, Epulis, foreign body, fungal, tumor, and epithelioid histiocyte types.
7. Endothelial cell	Innermost layer of the blood vessel wall	Regulating the passage of blood contents across the vessel Wall.	In contact with blood; controls the exchange of substances through the vessel wall; exists as continuous, fenestrated, and discontinuous types.
8. Neutrophil	Bone marrow, spongy bone, blood, lymph, and acutely inflamed tissues	Acting as the first line of defense; plays a role in acute inflammatory responses.	The most abundant is the neutrophilic granulocyte.
9. Eosinophil	Blood, bone marrow, subepithelial regions of the serous membranes in the digestive and respiratory systems	Defensing against parasites; involved in allergic responses.	It is distinguished by the presence of a bilobed nucleus and acidophilic granules.
10. Basophil	In parasitic infestations (in dogs with Dirofilaria immitis), their number in the blood increases.	Functions similarly to mast cells.	Contains histamine and heparin; rarely observed in circulation.

11. Platelets	Sites of vascular injury	Forming platelet plugs, contributing to vessel constriction and clot formation.	Platelets are anucleate cytoplasmic fragments of megakaryocytes in the bone marrow with an advanced tubular system; in avian, they are in the form of thrombocytes that form nucleated clusters with pale cytoplasm.
12. Megakaryocyte	Bone marrow	Production of platelets	They are large cells with abundant cytoplasm and multilobed nuclei, also found in the spleen of rodents.
13. Fibroblast / Fibrocyte	Connective tissue	Forming the extracellular matrix of connective tissue.	It is the primary cell of connective tissue types.
14. Mast cell	Connective tissues, particularly in the bone marrow	Playing a role in immunological and inflammatory responses by releasing histamine, heparin, and serotonin.	Its granules exhibit metachromatic staining properties.
15. Plasma cell	Loose connective tissues, secondary lymphoid organs	Producing antibodies	They differentiate from B lymphocytes. The pale Golgi zone in the perinuclear region is prominent.
16. Mesenchymal cell	Connective tissues are embryonic connective tissue cells.	Providing the formation of tissues developed from mesenchyme	They are stellar in shape and contain little cytoplasm, similar to fibroblasts.
17. Reticular cell	Forms a network and synthesizes reticular fibers in connective tissues.	Synthesing reticular fibers	It is found in lymphatic and hematopoietic organs.
18.Chondroblast	Inner layer of the perichondrium	Producing proteoglycans	Differentiates from mesenchymal cells.
19. Chondrocyte	Mature/adult cartilage tissues	Synthesing the fibers, amorphous ground substance, and chondronectin of cartilage	Their nuclei are more rounded than those of chondroblasts.

20. Osteocytes	Canaliculi ossei are found in bone tissue.	Maintaining the organic matrix and bone structure; regulates calcium release.	Possesses an oval or flattened nucleus; contains a poorly developed granular endoplasmic reticulum and Golgi apparatus.
21. Osteoblast	Found on the bone surface. It originates from osteoprogenitor cells.	Synthesing the fibers and glycoproteins of the organic matrix	It has a cuboidal-columnar shape and basophilic cytoplasm.
22. Osteoclast	Monocyte-derived; located in bone resorption areas (Howship's lacunae)	Performing bone resorption	It is activated by calcitonin stimulation and has acidophilic cytoplasm.
23. Osteoprogenitor cell	The inner cellular layer of the periosteum is found in the connective tissue of Haversian and Volkmann's canals, in the endosteum.	Forming osteoblasts	Plays a role in bone growth and fracture healing.
24. Purkinje's fiber cell	Arranged in bundles within the heart muscle	Conducting rhythmic impulses; distributed throughout the cardiac muscle	Cytoplasm appears pale due to high glycogen and mitochondrial content.
25. Satellite (mantle) cells	It is found in the connective tissue surrounding the ganglion cells in ganglia.	Supporting gangliosides; they are a special type of glial cells	It is found in single-layer arrays around the ganglioside.
26. Neuron	Principal cell of nervous tissue	Processing signals, makes decisions, contributes to memory and control	Found in various sizes and shapes within the gray matter of the central nervous system
27. Ganglioside	Found in ganglia	Enhancing the transmission of nerve signals; prevents their weakening.	They are large neurons surrounded by satellite cells in ganglia.

28. Microglia	Organs of the central nervous system	Performing phagocytosis; it is the resident macrophage of the central nervous system	Derived from blood monocytes that migrate into nervous tissue and transform
29. Astrocyte	Organs of the central nervous system	Continuous enveloping the central nervous system from the outside; providing nourishment for nerve cells and isolation from the external environment	Protoplasmic astrocytes surround nervous tissue externally, while fibrous astrocytes connect neurons and blood vessels within the tissue
30. Ependymal cell	It lines the wall of the central cavities of the central nervous system.	Contributing to the formation of cerebrospinal fluid	Located in an epithelioid manner on the wall of the central nervous system cavities.
31. Oligodendrocyte in the central nervous system (CNS)	Found in the white matter of the central nervous system	Contributing to the nourishment of neurons in the central nervous system and covers axons with myelin	Located along nerve fibers in the white matter
32. Schwann's cell in the peripheral nervous system (PNS)	It surrounds the axons in peripheral nerve fibers.	Enwraping axons and form myelin sheaths, contributing to the structure of the nerve fiber histologically	Arranged in sequences between axons
33. Pericyte	Envelops endothelial cells externally	Regeneration and repair	Surrounds blood vessels; derived from mesenchyme
34. Melanocyte / Melanophore	Melanocytes are located in the connective tissue beneath the epidermis, while melanophore cells are cells of the stratum basale layer of the epidermis and phagocytose melanosomes.	Producing melanin pigment	Melanocytes synthesize melanosomes containing melanin and release them extracellularly; basal epidermal cells that receive these form melanophore cells and determine skin color.

The number of cell types in the body is much higher than those given in table 2., exceeding 200. This is because in different tissues and organs, there are dozens of different subtypes of the basic cell types provided in the table. The subtyping of these different cells is done using special molecular techniques.

The formation of cells with the most appropriate shape, structure, location, and metabolism for their functions from the zygote, which is a cell of dual origin, occurs through the continuous differentiation of embryonic cells during the embryonic period (Sağlam et al., 2001; Şahin et al., 2005; Junqueiro & Carneiro, 2006; Seçkin et al., 2007; Sağsöz & Ketani, 2008; Eşrefoğlu, 2009; Matur & Solmaz, 2011; Girgin et al., 2016; Özer et al., 2021).

4. CURRENT APPROACHES IN CELL TYPING

Cells have traditionally been classified and typed based on their structure, shape, location, and certain special substances they possess, as observed through microscopes. Research on the brain cells, and the molecules they produce dates back to Santiago Ramón y Cajal and his contemporaries, who, in the 1930s, were able to reveal with high accuracy the organization and distribution of cell types across different regions of the brain (Ramón & de Castro, 1933; Zeng & Sanes, 2017; Yuste et al., 2020). These traditional cell identification methods are unfortunately no longer sufficient in scientific research or diagnostic methods under today's conditions, where digital imaging and analysis systems, molecular techniques, and automation are highly advanced. Therefore, intensive studies are being conducted on cell typing methods that are free from subjective errors and based on objective digital data. Some methods that have been quite successful in typing certain cell types have also yielded very important positive results in animal production, the most significant example being the separation of spermatozoa carrying X and Y chromosomes in bull sperm. This method is also successfully applied in humans (Otto et al., 1979; Pinkel et al., 1982; Garner et al., 1983).

It is quite difficult to determine the characteristics of a cell type and the sharp boundaries between different cell types, as even the cellular properties of a highly differentiated cell show a high degree of heterogeneity and significant variations are observed. Currently used techniques in cell typing and identification include many methods such as flow cytometry, immunofluorescence, morphological analysis, and molecular analysis. These techniques help identify and separate cells based on the nature of proteins on the cell surface, clusters of differentiation (CD) molecules, or genetic materials. These mentioned methods can also be used in cell separation. Cell separation is the process of segregating cells according to their different properties. These properties can include cell size, density, electrical charge, surface proteins, and gene expression. This separation process allows for the segregation of a group of cells selected according to their characteristics (Wilchek & Chaiken,

2000; Nunez, 2001; Zou et al., 2001; Cartron et al., 2002; Hage & Cazes, 2005; Ibrahim & Van Den, 2007; Polat & Karahan, 2009; Yoltaş & Karaboz, 2010; Çalışkan, 2019).

Techniques applied in cell separation and purification include many methods such as flow cytometry, magnetic separation, ultrasonic separation, and fluorescence-activated cell sorting (FACS). These methods help separate cells according to their specific characteristics and collect the separated cells (Garner et al., 1983; Prince et al., 1987; Johnson et al., 2007; Will & Steidl, 2010; Curtis et al., 2011; Tomlinson et al., 2013; Hoeve et al., 2016; Rodrigues et al., 2016; Rahmanian et al., 2017; Pathak & Banerjee, 2020; Okşak & Kuruca, 2021).

5. TECHNIQUES APPLIED IN DETERMINING CELL IDENTITY

In determining cell identity, techniques such as flow cytometry, immunohistochemistry, immunofluorescence, magnetic-activated cell sorting, affinity chromatography, FACS, gene expression analysis, bioinformatics analysis, cell chip, single-cell sequencing, density gradient centrifugation, laser microdissection are utilized. These techniques help identify and separate cells based on the properties of proteins or genetic materials found on the cell surface, cell densities, surface electrical charges of cells, and their motility.

5.1. Flow Cytometry

Flow cytometry is a method used to qualitatively and quantitatively examine the phenotypic and characteristic features of cells, and to identify and count individual cells. The device that operates using this method is called a flow cytometer. The flow cytometry system was developed to detect cellular structures by combining vital fluorochromes, monoclonal antibodies, and technologies based on labeling with fluorochromes. This device emerged through the integration of physics and computer technology (Zu et al., 2009). In flow cytometry, various cells pass individually through a flow channel in suspension, and during this process, they are classified according to cell size and granularity. Using this device, the analysis and differentiation of cell surface and cytoplasmic proteins, organelles, and other components are performed based on size, granularity, and fluorescence emission using laser and electronic technology (Nunez, 2001; Ibrahim & Van Den Engh, 2007).

Flow cytometry allows for the detection of different cell types (hematopoietic, lymphoid, or non-hematopoietic) or cell varieties (B and T cells, myeloid/monocytic cells, epithelial cells, natural killer cells, and neuro/neuroendocrine cells) or precursor and mature cells at different stages of cell maturation by using appropriate antibodies (Kim et al., 2008). If a flow cytometry device is to be used for analysis, various samples such as blood, spinal fluid, pleural fluid, bone marrow, bronchoalveolar lavage fluid,

cerebrospinal fluid (CSF), joint fluid (synovial), cell culture samples, and tissue biopsy samples can be used (Azkur et al., 2005; Rose & Knox, 2007; Peterson et al., 2008).

In flow cytometry, cells or particles in suspension are passed through a chamber illuminated by laser light. Each cell passing through this chamber is illuminated by the laser light, and the resulting emission wavelength rays are captured by appropriate detectors, filtered, and subsequently analyzed (Nunez, 2001; Ibrahim & Van Den Engh, 2007). Thus, flow cytometry enables obtaining highly sensitive data about various properties of a cell or particle. These data include pH changes in the cell cytoplasm, live-dead discrimination, immunophenotypes, enzyme activities, DNA content, and detection of marker molecules found on the cell surface, in the cytoplasm, and/or nucleus that are recognized by specific antibodies (Dunphy, 2004; Suvas et al., 2006; Zu et al., 2009).

Flow cytometry devices are extremely fast and provide detailed data by analyzing thousands of cells in a very short time. However, since these devices do not generate images, they cannot determine the morphological features of cell or tissue components in detail. Therefore, other imaging techniques such as fluorescence microscopy or confocal microscopy are needed to obtain more visual information about structural details.

Although the use of flow cytometry alone is not common in Veterinary Medicine, its combined form with cell sorting systems (FACS) is extensively used in separating bull spermatozoa carrying X or Y chromosomes. For this purpose, spermatozoon DNA is labeled with a vital fluorochrome. However, for flow cytometry to be more widely used in veterinary medicine, both the quantity and quality of markers used to identify cell characteristics in different animal species need to be increased. This can be possible through the commercial availability of relevant specific monoclonal antibodies at reasonable costs (Byrne et al., 2000).

5.2. Immune Techniques Applied in Cell Typing

The basis of these techniques relies on the principle of recognizing cell-specific membrane molecules, cytoplasmic organelles, or substances using monoclonal antibodies. Based on the labeling of the monoclonal antibody, these techniques are divided into 3 main groups: immunofluorescence, enzyme immunohistochemical techniques, and immunogold. In recent years, labeling with magnetic nanoparticles, which is mainly suitable for cell separation, has also begun to be applied.

5.2.1. Immunofluorescence

Immunofluorescence is a technique that involves conjugating specific molecules in a cell or tissue with monoclonal antibodies labeled with

fluorescent dyes (fluorochromes) and subsequently visualizing these markers using specially designed microscopes. The microscopes used for this purpose are primarily of two types: classical fluorescence microscopes and confocal laser scanning microscopes (CLSM). Fluorescence microscopes excite molecules with high-energy UV light of very short wavelength (<400 nm); the excited molecule emits photons at a longer wavelength according to Stokes' law. The wavelength of light that excites the molecule is called the excitation wavelength, while the wavelength of the emitted light is called the emission wavelength. In this process, the excitation filter selects light of a specific wavelength; it passes through a dichromatic mirror to illuminate the sample. The fluorescence emission light passes through a protective barrier filter before being transmitted to the observator's eye or photosensitive surface of the digital recording system. Currently, laser diodes are used instead of classical mercury lamps for more effective illumination. CLSMs develop this principle to obtain 3D images. In CLSM, laser light focuses on different virtual optical planes on the sample, creating images in specific layers along the Z-axis. These optical sections taken from different planes are combined to obtain 3D and time-variable images; thus, dynamic events such as cell surface molecules, apoptosis, cell division, and pH changes in the cytoplasm can be examined in detail. Hundreds of fluorochromes have been developed for these techniques, and both classical and confocal systems are extensively used for immunofluorescence studies today (Çalışkan, 2019).

5.2.2. Enzyme Immunohistochemistry

Enzyme immunohistochemistry has emerged from the combination of enzyme histochemistry and immunological techniques. In this method, monoclonal antibodies are conjugated with an enzyme, and subsequently, the enzyme histochemical method is applied to make the enzyme visible. It has significant advantages such as allowing examination with a conventional light microscope and obtaining detailed cell and tissue images, resulting in better localization of positive results.

This method can be used for tumor typing and differential diagnosis of diseases, detection of hormone receptors such as estrogen and progesterone, demonstration of certain genetic changes, and determination of cell proliferation rates. For these purposes, automated staining devices and labeled monoclonal antibodies have been developed.

5.2.3. Immunogold technique

Although the results can be visualized with a light microscope, it is essentially an immunological technique developed for electron microscopic examination. In this technique, the element that provides imaging is gold nanoparticles used in labeling monoclonal antibodies. Since the image in transmission electron microscopy (TEM) is formed based on whether

electrons pass through the examined structure or not, and in scanning electron microscopy (SEM) by reflection from the sample surface, fluorochromes cannot be used for labeling antibodies. Therefore, monoclonal antibodies are labeled with gold nanoparticles for use in electron microscopes (Ateş, 2018).

6. CLUSTER OF DİFFERENTIATION (CD) MOLECULE FAMILY

These molecules are membrane molecules (surface antigens) expressed on the cell membrane surface during the process of cell differentiation from the embryonic period onwards. This family of cell membrane molecules, which will be widely used in cell typing in the near future, was named cluster of differentiation (CD).

6.1. CD Molecule Familiy

The major histocompatibility complex (MHC), found on the membrane surfaces of the cells involved in the adaptive immune system, is a large family of cell membrane proteins. As they were first detected in leukocytes, they are also called human leukocyte antigens (HLA). In humans, the gene region encoding this system is located on chromosome 6. It is a molecule family that particularly utilized in determining tissue compatibility characteristics in tissue and organ transplantations. These molecules mediate the interaction of neutrophils with other white blood cells and other body cells. Therefore, MHC determines the compatibility of the donor with the recipient in organ transplants and an individual's susceptibility to autoimmune diseases. The MHC system was first described by Peter Gorer in 1936. In subsequent years, the system's functions were determined in more detail, and the gene sequence encoding the human MHC system was identified in 1999. Shortly thereafter, MHC gene sequences from other species were also mapped. MHC I antigens are predominantly found in CD8-positive T-lymphocytes, while MHC class II (MHC II) antigens are found in antigen-presenting cells (APCs) and CD4-positive T-cells (Bernard & Boumsell, 1984; Cobbold & Metcalfe, 1994; Lunn et al., 1996; Saalmüller et al., 1996; Sopp et al., 2001; Zola et al., 2005; Kierszenbaum, 2006).

6.2. Identification of CD Molecules

The expression of MHC complex components in multiple different cells complicates the use of this molecular family in typing individual cells. Therefore, intensive work is being conducted on utilizing the cluster of differentiation (CD) molecule family, especially in immunophenotyping of immune system cells. The CD nomenclature system was first addressed at the 1st Human Leukocyte Differentiation Antigen (HLDA) workshop in Paris in 1982. The primary aim of this meeting was to categorize different monoclonal antibodies (mAbs) produced in various laboratories worldwide (Bernard & Boumsell, 1984).

This terminology was created to standardize the classification of cell surface antigens and to identify molecules expressed by different hematopoietic cell types (Cobbold & Metcalfe, 1994; Lunn et al., 1996; Saalmüller et al., 1996; Sopp et al., 2001).

Although the structural and functional relationships between the MHC system and the CD system have not yet been fully elucidated, some preliminary information has been obtained. For example, the invariant chain, also known as Ii/CD74, has been identified as a non-polymorphic MHC II integral membrane glycopeptide that acts as a chaperone in stabilizing newly synthesized MHC II heterodimers. Furthermore, during T cell activation, CD4 and CD8 molecules form a bridge with the T cell receptor (TCR). Due to this close relationship, CD4 and CD8 are referred to as co-receptors of MHC, which is a multimolecular complex, and are considered an integral part of this system.

In the CD system, cell surface molecules recognized by monoclonal antibodies are characterized as antigens. These antigens are special markers that enable specific identification and typing of cells. These CD molecules are primarily cell surface molecules expressed on the cell membranes of leukocytes and other cells of the immune system, and these molecules recognize a specific member of a cell group and other members of the group (Zola et al., 2005; Kierszenbaum, 2006). For example, this allows a helper T lymphocyte expressing CD4 to be distinguished from a killer T cell expressing CD8. CD markers enable the classification of T cells involved in inflammation and immune reactions. CD antigens initiate signals that mediate cell-to-cell interactions, adhesion, and T cell activation (Kierszenbaum, 2006).

Significant studies have been conducted using specific mAbs to determine the cross-reactions of CD antigens of human and animal cells, aiming to identify the counterparts of cell types from different species in other species (Cobbold & Metcalfe, 1994; Lunn et al., 1996; Saalmüller et al., 1996; Sopp et al., 2001). During classification, mAbs with similar reactivity in different species are assigned to a cluster molecule group (e.g., CD4 mAb), and an antigen recognized by a cluster of mAbs is assigned a "cluster of differentiation (CD)" number (e.g., CD4 molecule/antigen). In identifying cell populations, '+' or '–' symbols are used. For example, a "CD34+, CD31–" cell expresses CD34 but does not express CD31. The expression intensity of these molecules is classified as high (hi), middle (mid), or low. The small "w" preceding the number definition means "workshop" and indicates at which numbered meeting this temporary number was given (e.g., CDw12); it denotes an antibody or molecule that has not been sufficiently characterized. In some cases, if mAbs defining only one antigen cluster are obtained from the same laboratory, the suffix "w" is added to the CD definition (Zola et al., 2005; Saalmüller & Aasted, 2007).

Capital letters following a CD number indicate an added variant of the extracellular domain of a cell surface molecule. For example, CD45RA or CD45RO correspond to additional variants of CD45. A lowercase letter following the CD number (e.g., CD1a, CD1b, CD1c, CD1d, or CD1e) indicates several molecules sharing a common chain. In other cases, lowercase letters have been used to name different members of the same gene family, as in CD66 (CD66a, CD66b, CD66c, CD66d, CD66e, and CD66f). Regarding carbohydrate CD structures, a lowercase suffix represents a modification of the same carbohydrate sequence (Zola et al., 2005).

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Chapter 2

USAGE OF NANOBOTS IN MEDICAL FIELDS

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1.Introduction

Over the past decade, micro- and nanoscale devices have attracted increasing interest in various fields such as monitoring, decontamination, and biomedical applications (Yang 2020). In this context, nanorobots constructed from diverse materials and structures demonstrate significant potential in disciplines including detection, purification, and micromanufacturing. Owing to their superior mobility, these systems are capable of actively binding to target molecules and enhancing mass transfer within solutions, which renders them particularly advantageous for biosensing applications (Kong 2018).

Although conventional motor and gas-propulsion systems are effective at the macroscopic scale, their miniaturization to sub-millimeter dimensions poses significant manufacturing challenges and leads to reduced performance. As an alternative to these limitations, nanobots stand out by offering advantages such as natural structuring at the microscopic scale, high energy efficiency, flexibility, and versatile mobility (Lin, 2021). Biomedical applications of micromotors demand rapid and precise performance in confined spaces and highly viscous fluids. Advances in understanding the operation of micromotors within body fluids and living organisms have enabled applications such as targeted drug or cell delivery (Striggow, 2020).

2.Nanobots

To enable nanorobots to perform specific tasks, various propulsion mechanisms have been explored. These include self-propelled systems (chemical-based), propulsion methods driven by external stimuli (such as magnetic fields, light, acoustic waves, ultrasound, or electric fields), as well as biohybrid strategies utilizing bacteria, viruses, sperm, and cells. With their abilities to swim, rotate, flow, and penetrate target cells or tissues, these devices can be applied in vivo when combined with appropriate imaging techniques (Agrahari, 2020). However, for specific tasks to be accomplished in a controlled and targeted manner, guidance capability emerges as a fundamental requirement (Hormigos, 2018).

Although synthetic nanomotors possess remarkable capabilities such as exceptional speeds and high cargo-carrying capacity, their potential applications are severely constrained by certain limitations. These include a lack of biocompatibility, the toxic effects of the chemical fuels employed, and the need for specialized equipment for external guidance (Filatov, 2019).

To mitigate and ultimately overcome these limitations, recent efforts have focused on the design and development of biohybrid nanomotors—composed of both artificial and biological components—that emulate the characteristics of living organisms (Li 2019).

	Key features	Advantages	Limitations
Self-propulsion	Use chemical reactions to generate bubbles or chemical gradients for propulsion	High swimming speed when the fuel is adequate; Low cost	Continuous fuel supply is needed in the microenvironment for powering; Most of the fuels are toxic; Lack of directionality
External propulsion	External power provided by magnetic fields, light, acoustic fields, electric fields, etc., for actuation	Position can be precisely controlled; Good directionality	Special manipulating equipment is usually required.
Hybrid actuation strategy	Combination of different actuation strategies, including self-powered strategies, externally-powered strategies, and motile live cells (e.g., sperm cells) or microorganisms	Relatively efficient in power output; Good control on directionality; Responsive to multiple stimuli	Live cells survive only under certain conditions; Precise control of the live cells might be difficult

Table 1: *Classification According to Propulsion Power Source (Chen 2018)*

2.1. Self-Propulsion

Self-propelled micro/nanomotors have attracted significant attention not only due to their simple geometries and straightforward fabrication techniques, but also because they serve as fundamental models for investigating the complex behaviors of more sophisticated micro/nanomotors and hold promising applications across various fields (Yang 2020).

2.1.1. Bubble-Based Propulsion System

Bubble-propelled nanomotors are designed based on a simple principle: propulsion is achieved through the generation of numerous bubbles, which occur either from the catalytic decomposition of a chemical reactant such as hydrogen peroxide (H_2O_2) under suitable conditions, or from gas-producing redox reactions (Liang 2017). The diverse geometries of nanomotors also influence their mobility. Notably, the design of bubble-propelled nanomotors increasingly incorporates specific geometrical features to achieve more controlled motion (Su 2019).

Bubble-propelled nanomotors, characterized by strong propulsion force and high biocompatibility, are regarded as highly promising tools for biomedical applications, particularly if precise control can be achieved and propulsion systems can be made more durable (Yang 2020).

2.1.2. Electrophoresis-Based Propulsion System

Electrophoresis refers to a self-propulsion mechanism observed in biological systems, occurring in aqueous environments through ion exchange between microorganisms and their surrounding solutions (Mitchell 1972). The motion of these nanomotors is driven by the conversion of chemical energy present in their surrounding environment into mechanical energy (Nourhani 2015).

In simple terms, a conductive microparticle generates an electric field as a result of redox reactions occurring at its two ends. Oxidation at the particle's anode produces an electron flow, which is consumed at the cathode through a reduction reaction. Simultaneously, protons are generated at the anode and consumed at the cathode. The migration of these protons drags the surrounding fluid along, propelling the microparticle in the opposite direction (Paxton 200

Although self-propelled electrophoresis nanomotors offer advantages over bubble-propelled motors in terms of motion control, they also present notable limitations. These motors typically possess insufficient propulsion energy and cannot remain active for extended periods. Moreover, most of the solution environments employed (such as H_2O_2 or hydrazine) exhibit toxic properties, restricting the use of these motors in pharmaceutical and biomedical applications (Yang 2020).

6).

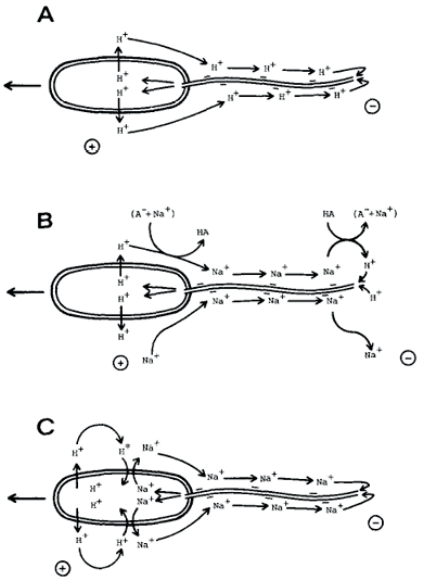

Figure 1: *Self-Electrophoretic Bacterial Propulsion Mechanism*

2.1.3. Surface Tension Gradient-Based Propulsion System

Differences in solvent concentration create interfacial energy disparities, resulting in a surface tension gradient between two liquids. This gradient induces mass flow capable of propelling objects at the micrometer and nanometer scale, a phenomenon known as the "Marangoni Effect" (Chen 2015). Nanomotors driven by surface tension gradients can exhibit chemotactic behaviors, moving in predetermined directions within a liquid environment (Plutnar and Pumera 2018).

Unfortunately, most motors propelled by surface tension gradients are far from being truly "nano" in scale. Consequently, recent studies have focused less on enhancing the mobility of these motors and more on reducing their size (Yang 2020).

2.2. External-Propulsion

Externally powered micro/nanomotors have attracted significant attention not only due to their simple geometries and straightforward fabrication techniques, but also because they serve as fundamental models for studying the complex behaviors of more sophisticated micro/nanomotors and possess promising applications across various fields (Yang 2020).

2.2.1. Light-Driven Propulsion System

Light is a widely available and renewable energy source that can be transmitted remotely with ease (Gorostiza and Isacoff 2008). The concept of light-driven nanomotors, first introduced by Jean-Marie Lehn in the 1980s, is fundamentally based on propulsion generated through photochemical reactions, photothermal effects, or photochromic processes. These approaches hold the potential to eliminate the reliance on toxic chemical fuels (Yang 2020).

The availability of different light intensities and wavelengths has enabled the use of a wide range of materials, designs, and environments (Chen 2018). Although most light-driven systems rely on short wavelengths due to their high energy-carrying capacity, certain systems successfully employ other wavelengths such as IR (infrared), UV (ultraviolet), and NIR (near-infrared) to propel responsive nanomotors, making them more suitable for biomedical applications (Hormigos 2018) (Sonntag 2019). However, the efficient transfer of light energy to nanomotors in opaque environments remains a significant challenge. Conventional UV and NIR light sources can penetrate only a few millimeters within biological tissues, making them insufficient for reaching deeper regions (Xu 2019). X-rays, a widely available light source with adequate penetration capacity, offer a potential solution to this limitation and additionally allow remote control of propulsion speed through variation of radiation dose (Yang 2020).

2.2.2. Electric Field-Driven Propulsion System

Electric field propulsion mechanisms, distinguished by their low cost and ease of integration, are considered one of the most promising fuel-free propulsion strategies for nanomotors (Deng 2018). When particles are exposed to an electric field, electrokinetic effects arise, which can be harnessed to facilitate their motion (Chen X. Z. 2018).

Although the use of direct current (DC) electric fields offers certain advantages, alternating current (AC) electric fields are more commonly employed to eliminate uncontrolled electrophoretic effects (Yang 2020). In the AC operating principle, the constant electric field established between electrodes generates electroosmotic flow, which not only drives the diodes but also pumps the surrounding fluid. Throughout this process, the diodes suspended at the liquid surface move parallel to the electric field (Chen X. Z. 2018).

2.2.3. Magnetic Field-Driven Propulsion System

In recent years, magnetically driven nanomotors have attracted considerable attention, particularly in biomedical and pharmaceutical fields, due to their fuel-free operation, remote controllability, and non-destructive interaction with cells and tissues (Chen X. Z. 2018). Another advantage of employing magnetic fields is the ability to utilize the components of the nanobot as micro/nano drills. This configuration enables the drills to perform incisions on cells and tissues, thereby allowing each nanobot to be employed in microscale surgical procedures (Magdanz 2014).

Certainly, propelling nanobots alone is not enough; their movement within in vivo environments must also be monitored. This requires integrating magnetic propulsion with an appropriate imaging technique. Among the available methods, ultrasound emerges as a particularly promising option for tracking nanobots, owing to its non-invasive approach, absence of radiation, and ability to provide real-time imaging (Middelhoek 2022). Although magnetically driven nanomotors have garnered increasing interest and a substantial number of studies have been published recently, the development of these technologies remains in its early stages. Their successful translation into widespread biomedical applications faces significant challenges, particularly due to the lack of precise fabrication methods and the limited biodegradability of magnetic materials (Yang 2020).

2.2.4. Sound-Driven Propulsion System

Recent studies have shown a marked increase in interest toward systems that utilize acoustic fields as a propulsion mechanism (Chen X. Z. 2018). The term acoustic propulsion primarily refers to ultrasonic waves with frequencies ranging from 20 kHz to several gigahertz. Ultrasonic waves can be directed,

confined, and focused, making them suitable for propelling nanomotors (Sonntag 2019). Fundamentally, the control of movement is achieved directly by adjusting the intensity of the acoustic or ultrasonic field (Yang 2020).

A distinctive advantage of acoustic-based systems over other field-driven approaches is their ability to independently activate and propel each micro-swimmer within a group. This capability serves as a powerful tool for executing cooperative functions (Chen X. Z. 2018). Although acoustic propulsion offers advantages such as high-speed potential and biocompatibility, the ability to precisely guide nanorobots within living organisms remains limited. Certain shortcomings, including the inability to optimize these systems fully and the unpredictability of micromotor behavior in practical settings, may restrict the widespread application of acoustically driven micromotors (Agrahari 2020).

	Magnetic fields	Light	Acoustic waves	Electric fields	Thermal
Speed	++	+	+++	+	+
Directionality	+++	++	++	+	+
Positioning precision	+++	+++	+	++	+
Dimensions of motion	3	3	2	2	2
Feasibility for biomedical application	+++	+++	++	+	+
Complexity of control system	+++	++	+	+	+

Table 2: *Comparison of Different Propulsion Mechanisms in Externally Powered Nanomotors (Chen 2018)*

2.3. Biohybrid Nanomotors

Inspired by nature, biological microswimmers such as bacteria and sperm can be directly combined with small-scale synthetic robots, giving rise to a specialized class of nanorobots known as biohybrid nanorobots. This integration enables the incorporation of biological motility into engineered systems (Singh 2020). Furthermore, the integration of motile microorganisms into microsystems has demonstrated significant potential for the development of micro-biorobots (Magdanz 2013). This potential stems from factors such as high compatibility with biological systems, functionality under physiological conditions, efficient locomotion at the microscopic scale, the ability to interact with living structures, and the capacity for specific interactions with certain biological processes (Sonntag 2019). Hybrid systems, particularly those utilizing bacteria and sperm cells, have shown significant promise for transporting microscale cargos and drugs to specific targets in both in vitro and in vivo settings (Striggow 2020).

Types of biohybrid micro/nanomotors	Motor type	Propulsion source and motion control	Potential applications
Sperm hybrid	Sperm cell with metal-coated polymer microhelices	Sperm cell and magnetotactic control	Assisted reproduction
	Bovine sperm cell micromotors	Sperm cell and magnetotactic control	Drug delivery
	Functionalized sperm micromotors loaded with nanoscale synthetic payloads	Sperm cell and chemotactic control	Drug delivery
Bacteria hybrid	bacteria-driven microswimmer	Bacteria-driven and chemotactic and magnetotactic control	Drug delivery
	Janus fiber rods	Bubble with catalase as fuel and geometries	Bacteria detection
	Bacteria-driven spherical microbeads	Bacteria-driven and chemotactic control (size and geometries)	Cargo delivery
	Cell-membrane-coated bacteria	Blood circulation	Tumor imaging
Cell hybrid	Red blood cell-mimicking micromotor	Ultrasound energy and magnetotactic control	Oxygen transportation
	Platelet-camouflaged nanorobots	Magnetic propulsion and magnetotactic control	Isolation of biological threats
	Macrophage–Mg biohybrid motors	Hydrogen bubble propulsion	Endotoxin neutralization
	Neutrophil-based micromotors	Cell-driven and chemotactic control	Target drug delivery
Enzyme-propelled	Enzyme-powered microshell motors	Catalase-trigged bubble propulsion and chemotactic control, size	Drug delivery
	Mesoporous silica-based nanomotors	Urease-powered and pH responsive	Target drug delivery
	Micromotors equipped with DNA nanoswitches	Urease-powered and pH responsive	Microenvironment sensing and micromotor activity status indicator
	Ultrasmall stomatocyte motors	Biocatalyst catalase and chemotactic control	Drug delivery

Table 3: *Types of Biohybrid Nanomotors (Yang 2020)*

2.3.1. Sperm Cell-Based Hybrid Nanomotors (Spermbots)

One of the significant advancements in reproductive science is the development of nanorobotic systems designed for the manipulation and selection of sperm cells. The ultimate goal of these developments is to enhance sperm functionality (Benhal 2024).

Numerous studies to date have focused on the design and fabrication of sperm cell-based nanorobots. Some of these studies aimed to directly harness the natural swimming ability of motile sperm cells as a propulsion source; this biological motility, when integrated with magnetic guidance systems, has enabled the development of biohybrid microswimmers (Celi 2021). Consequently, the concept of the "spermbot," a motorized system intended for use in assisted reproductive technologies such as artificial insemination and in vitro fertilization (IVF), has emerged (Singh 2020).

One of the advantages of using sperm cells to propel biohybrid nanobots is that these cells are readily obtainable, practical to use, can be employed directly without the need for culturing, and are completely harmless. Furthermore, spermbots, which are naturally adapted to swim in high-viscosity environments such as serum (Magdanz 2013), can be integrated with materials that confer magnetic properties, allowing both their orientation and direction of movement to be controlled with high precision using external magnetic fields (Singh 2020).

2.3.1.1. Microtubules

One biohybrid approach utilizing sperm cells involves confining motile cells within microtubes; within this structure, the cell generates a propulsive force on the surrounding fluid, while the microtube acts as a magnetic rudder for directional control (Magdanz 2020). Since microtubes are too large to be internalized by the cells, their interaction with sperm does not adversely affect sperm motility or the ability to undergo the acrosome reaction (Magdanz 2013). The speed of the spermbot is determined by the degree of confinement of the sperm flagellum within the microtube. Shorter microtubes allow the flagellum greater freedom of movement, enabling more effective utilization of its natural amplitude, whereas longer microtubes restrict this motion, reducing the propulsive force (Magdanz 2015).

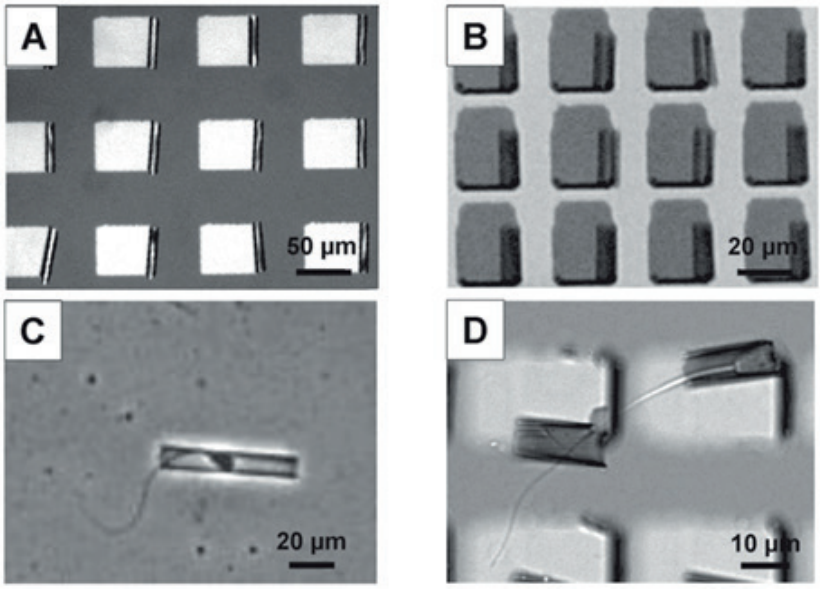

Figure 2: *A) Arrays of coiled microtubes with a length of 50 µm and B) 20 µm, both with a diameter of 7 µm. C) and D) depict a bovine spermatozoon captured within coiled microtubes of 50 µm and 20 µm in length, respectively (Magdanz 2015).*

2.3.1.2. Iron Sperm

Another approach, serving as an alternative to microtubes for guiding biohybrid nanobots propelled by the natural swimming behavior of sperm cells, is the "iron sperm" method. In this technique, the flexible structure of sperm cells is coated with magnetic particles, enabling magnetic propulsion while also enhancing cell echogenicity for ultrasound imaging (Middelhoek 2022).

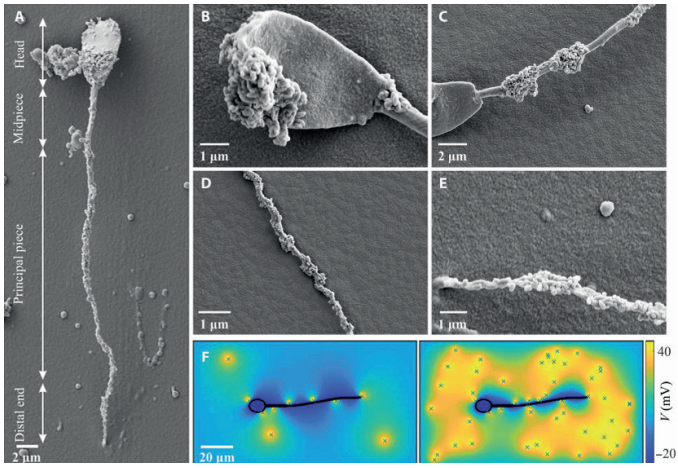

Figure 3: *A) Shows a bovine sperm cell coated with iron oxide particles. B) Sperm head, C) midpiece, D) principal piece, and E) the tip of the sperm cell where the nanoparticles are attached. F) The density of the nanoparticle coating was determined based on the charge balance between the sperm cell and the nanoparticles (Magdanz 2020).*

2.3.1.3. Microhelices

Beyond microtubes and iron sperm applications, various studies inspired by the corkscrew-like propulsion mechanism of E. coli bacteria have developed helical nanorobots that can be actuated using rotating magnetic fields (Celi 2021). To mimic the screw-like motion of sperm flagella, microhelices compatible with sperm cell dimensions have been fabricated. A metallic helical structure, designed to wrap around the sperm flagellum, exhibits forward and backward screw-like movements under rotating magnetic fields. When the sperm reaches the egg, the direction of the external magnetic field is reversed, causing the metallic structure to retract and detach from the sperm, completing the spermbot release (Singh 2020).

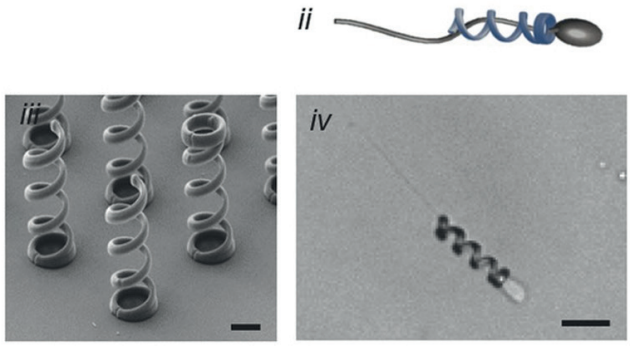

Figure 4: *Helical spermbot concept designed for the transport of immotile sperm cells. Scale bar: 10 µm (Singh 2020).*

2.3.2. Bacteria-Based Hybrid Nanomotors

Recently, numerous studies have focused on the development of bacteria-based hybrid nanomotors, leveraging the natural ability of bacteria to sense various environmental cues such as chemotactic attractants/repellents, pH, oxygen levels, temperature, and magnetic fields. These unique sensing capabilities of bacteria are particularly promising for guiding the movement of biohybrid microsystems in potential applications, such as targeted cargo delivery within complex biological environments (Yang 2020).

Bacteria can maintain their swimming ability in response to environmental gradients, allowing them to be integrated with various functional components for targeted navigation. For example, a single *E. coli* bacterium has been trapped inside a microtube produced via electrochemical methods; the inner surface of the tube was coated with a polydopamine (PDA) layer to attract the bacteria, while the outer surface was coated with nickel to enable magnetic guidance (Chen X. Z. 2018).

Another notable advantage of using bacteriabots lies in their ability to adhere to epithelial cells. This mechanism is naturally employed by bacteria to infect the urinary or intestinal systems (Sonntag 2019). Although bacteria-based nanobots hold significant potential, particularly for biomedical drug delivery applications, challenges such as immune responses (immunogenicity) and low motility performance remain critical obstacles that need to be addressed (Lin 2021).

2.3.3. Cell-Based Hybrid Nanomotors

Another promising type of biohybrid motor is the cell-based nanomotor. These motors can be constructed either by coating artificial nanomotors with cell membranes or by directly utilizing specific cells, such as red blood cells or neutrophils. Both approaches provide effective mobility within biological fluids while protecting the micromotors from biological fouling and clearance by the immune system (Yang 2020). Red blood cells can be loaded with magnetic nanoparticles and integrated into biohybrid nanomotor systems. This configuration enables both magnetic guidance and ultrasound-driven propulsion, while simultaneously reducing toxicity by encapsulating the drug within the blood cells (Sonntag 2019). Considering recent advances in research, cell-based biobots are regarded as highly promising propulsion sources. However, they also have certain limitations, including a finite lifespan, dependence on culture conditions such as nutrients, oxygen, temperature, and pH, and the need for specific requirements to function properly in in vivo environments (Lin 2022).

3. Conclusion

Advances in the field of "smart" micro/nanomotors—driven by synthetic chemistry, biochemistry, and inspiration from living materials—are paving the way for significant progress across related disciplines (Yang 2020).

As in other areas of medicine, the application of nanotechnology is expected to yield positive and supportive outcomes for patients. Therefore, researchers should be encouraged to conduct further in vitro and in vivo studies using animal models to assess the reliability and efficacy of these novel approaches (Remiao 2018).

Although current artificial nanorobots exhibit only rudimentary behaviors due to their relatively simple designs, maintaining this simplicity in future designs is desirable. Considering the large-scale production and implementation costs of these micromachines, avoiding unnecessary complexity makes their deployment more feasible and economically viable (Plutnar and Pumera 2018).

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Chapter 3

PSEUDOTUBERKILOSIS IN SHEEP AND GOATS

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Introduction

Sheep and goats are commonly affected by the chronic and infectious disease known as caseous lymphadenitis (CLA) (Ivanovic et al., 2009). The gram-positive, facultative intracellular, non-encapsulated, non-motile, fimbriated bacteria Corynebacterium pseudotuberculosis (C. pseudotuberculosis) is the cause of the diseease (Connor et al., 2000). A strong phospholipase-D exotoxin and a cell wall rich in mycolic acid are the two primary virulence factors of C. pseudotuberculosis (Bernheimer et al., 1985). Clinical manifestations of CLA may include visible superficial abscesses or signs of internal organ involvement, either alone or in combination, according to Dorella et al. (2006) and Al-Gaabary et al. (2009).The key to the effectiveness of management strategies is the identification of infected animals (Menzies et al., 2004). Clinical diagnosis can only be suggestive. This is since numerous bacterial species, aside from C. pseudotuberculosis, can cause superficial abscesses in small ruminants. The herd is typically affected by CLA lesions, although abscesses brought on by other pyogenic bacteria are more frequent (Baird and Fontaine, 2007). The most effective confirmatory diagnostic technique for CLA is the isolation and identification of C. pseudotuberculosis. The lack of isolation and the inability to reach visceral lesions for sampling, however, are two major problems with these diagnostic methods (Al-Gaabary et al., 2010). C. pseudotuberculosis can be identified serologically, which helps with the detection of subclinical infections. The most popular serological test for identifying an immunological response to C. pseudotuberculosis is the enzyme-linked immunosorbent assay (Hoelzle et al., 2013). The specificity and sensitivity of many serological assays to identify C. pseudotuberculosis infection are still up for discussion, though.Colonic identification or direct detection of C. pseudotuberculosis in pus samples may be possible with the genomic diagnosis of CLA by PCR. (Pacheco and others, 2007). Such a technique for identifying CLA instances is hampered by the inaccessibility of visceral lesions for collection and the dubious outcomes of PCR on blood samples. The location and extent of CLA visceral lesions have been extensively assessed in recent years using techniques including radiography and ultrasound (Oreiby, 2015).

In sheep and goats, C. pseudotuberculosis infects the superficial and visceral lymph nodes, resulting in internal or external infections. Lymph nodes, which also operate as filters to detect foreign items, are where immune cells are activated. Targeting lymph nodes, bacterial infections seek to weaken the host's defenses against the development of disease (Burmayan and Brundage, 2021). Necrosis, unchecked cell death, and the formation of highly apparent skin abscesses are all symptoms of affected superficial lymph nodes. The pus-like discharge from abscesses is the consequence of inflammation caused by bacteria that damage lymph node tissue. Because inflammation attracts neutrophils and spreads bacteria to other locations, animals can

indirectly spread the disease. In this respect, the internal organ lymph nodes that surround the lungs, liver, and spleen are somewhat more affected.

This article provides information on the history, etiology and epidemiology of Pesudotuberkilosis disease, as well as disease symptoms, pathogenicity, vaccination and treatment methods.

The Disease's History and Geographical Distribution

As a result of various studies on this organism, the organism began to be recognized as Bacillus pseudotuberculosis. The continuous search for a second name till the end of the 19th century was prompted by the idea that Mycobacterium pseudotuberculosis and caseous nodules shared clinical traits. The organism was renamed to be acknowledged as Corynebacterium ovis after being approved as belonging to the genus Corynebacterium with some names in various studies (Osman et al. 2018). Since the second name was specified for specification and completely missed the general ability that could imply other mammalian species as a host for the same agent, the species name ovis was returned to the previous pseudotuberculosis definition. Since then, C. pseudotuberculosis was officially determined to cause caseous lymphaditis (Komala et al. 2008).

The geographic distribution of caseous lymphadenitis and small ruminants is very similar. The disease is prevalent in the Middle East, South and North America, Africa, Australia, and Europe, and it usually affects several continents (Fontaine and Baird, 2008). The majority of these nations view CLA as a debilitating illness that causes financial losses in addition to other health issues pertaining to animal welfare in general. Nonetheless, the disease has primarily been found to afflict sheep and goats (Figure 1). A common source of infection is sufficiently supported by the tight genotypic associations between sheep/goat C. pseudotuberculosis isolates from different nations worldwide.It is thought that the disease spread after colonial powers exported sheep in the 18th century (West et al. 2002). Worldwide, new, stringent laws pertaining to the occurrence of lesions on imported corpses were put into effect. Several studies on different aspects of the disease, including pathogenesis and epidemiology, led to further investigation of control strategies identified to reduce the significant CLA prevalence at that time, and therefore preventive approaches were developed for future studies.

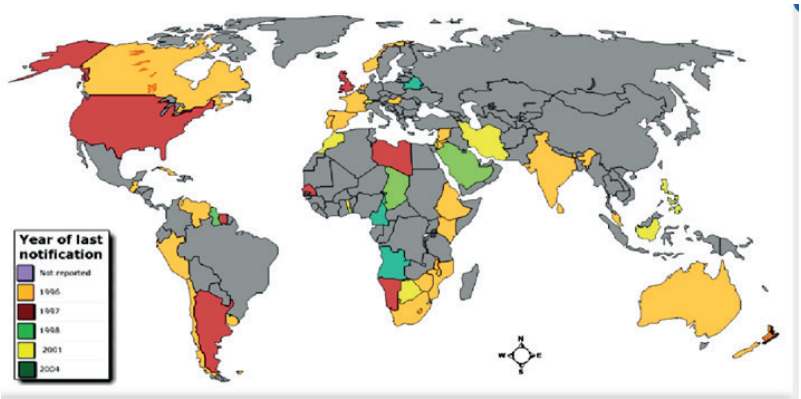

Figure 1. *The status of Caseus lymphatics disease in the world between 1996-2004*

Source: https://www.semanticscholar.org/paper

Microbiological Characteristics

In 1888, Nocard isolated C. pseudotuberculosis from cattle excrement. In 1894, Preisz was the first to thoroughly characterize this microbe and note how it resembled the diphtheria bacillus. Corynebacterium ovis, Preisz-Nocard bacillus, Bacillus pseudotuberculosis ovis, and Bacillus pseudotuberculosis are synonyms for C. pseudotuberculosis. (Hodgson et al. 1999). With sizes ranging from 1.0 µm to 3.0 µm and 0.5 µm to 0.6 µm, respectively, filamentous rods and coccoids are among the pleomorphic forms of this facultative intracellular pathogen. This bacterium is not motile, encapsulated, or spore-forming, although it does have fimbriae. Because it is a facultative anaerobe, the bacteria grows best at 37 °C and pH 7.0 to 7.2 (Oreiby et al. 2014).It first develops sparsely on the agar surface before forming palisades or clusters. It turns from cream to orange later. Colonies have concentric rings, are dry, and are opaque. According to Li et al. (2018), growth in liquid medium takes the form of a granular deposit with a surface membrane. Even though hemolysis varies in blood agar, Rhodococcus equi causes huge regions to form. Staphylococcal β-lysine's action is inhibited by C. pseudotuberculosis toxin (Farias et al. 2018). Volutin granules are visible when C. pseudotuberculosis is stained with Gram-positive stain using the Albert or Neisser procedure. Coccoid cells, which have high-energy phosphate reserves, lack these metachromatic granules, which are plainly visible in the bacillus form (Abdolmaleki et al. 2019).

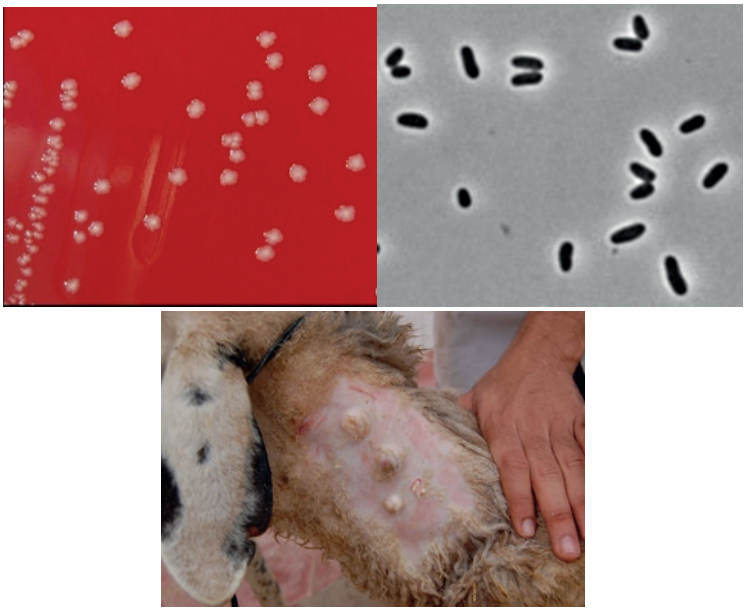

Figure 2. *Microbiological structure of C. pseudotuberculosis*
Source: https://mechpath.com/2017/12/13/corynebacterium-pseudotuberculosis/

Etiology

Gram-positive and facultative, C. pseudotuberculosis is an intracellular coccobacillus. Based on the bacteria's capacity to decrease nitrate, two biotypes have been identified. These include a nitrate-positive group that infects horses and a nitrate-negative group that infects sheep and goats. Cattle-derived C. pseudotuberculosis is a diverse group (Abdulrahman et al. 2020). All strains produce phospholipase D, an exotoxin that destroys endothelial cells and improves vascular permeability, which aids in the spread of the bacteria. A second virulence factor of C. pseudotuberculosis is an outer lipid coating that protects host phagocytes from hydrolytic enzymes. The bacteria multiply inside phagocytes before being lysed and expelled. The ongoing process of bacterial proliferation, which is followed by the withdrawal and eventual death of inflammatory cells, produces the characteristic abscesses associated with CL (Ruiz et al. 2020). C. pseudotuberculosis needs to pierce the skin or mucosal membranes in order to cause infection. The most common site of entrance is the skin after an injury, which may be caused by tail docking, castration, tagging, cutting, or other environmental risks. Contact with purulent material that drains from open, active lesions is the most common way for germs to enter these skin fissures. Another, though less frequent, way that C. pseudotuberculosis infections spread is through bacterial ingestion or inhalation that penetrates mucosal membranes. After entering the body, the

germs travel through the regional drainage lymphatic system to the lymph nodes. Internally, the germs infect internal organs in addition to lymph nodes. Encapsulated abscesses develop as a result of the incubation phase, which lasts one to three months (Costa et al. 2020). Additionally resilient to harsh environmental conditions, C. pseudotuberculosis can survive for eight months in soil and two months in fomites like bedding and wood. The creature has a better chance of surviving when there is organic matter, shade, and moisture present. In sheep and goats, caseous lymphadenitis (CLA) is brought on by the facultative intracellular gram-positive bacterium Corynebacterium pseudotuberculosis. Slow development, a pleomorphic shape, and the synthesis of exotoxins such phospholipase D (PLD) are characteristics of the pathogen that contribute significantly to its virulence. This bacterium mostly affects the lymphatic system, causing internal and superficial lymph nodes to develop abscesses. Typically, infection happens through mucosal surfaces and skin wounds from shearing, numbering, or other animal control techniques. Dry and dusty conditions are ideal for the bacterium's growth and survival. It is extremely contagious within flocks and can live in contaminated bedding, equipment, and soil (Taha, 2022).

Epidemiology

For 24 to 48 hours, C. pseudotuberculosis grows well on blood agar at 36°C. It produces tiny, opaque, whitish colonies that are pinpoint-sized and encircled by a weak hemolysis zone. The high lipid content of the bacterial cell wall, particularly corinomycolic acid, causes colonies to burn and disperse throughout the surface of the agar. The organism's ability to survive in macrophages may be aided by the high lipid content (Correa et al. 2018). Reports indicate that the disease is usually transcontinental and prevalent in most sheep-raising countries. However, epidemiological studies to determine sickness prevalence rates have only been conducted in a few countries globally; most of this research focuses on farms and slaughterhouses. According to Gallardo et al. (2019), the mean prevalence of CLA in adult sheep in flocks under study in Australia was 58% in 1973 and 53% in 1984. Statistics from one abattoir showed that 54% of adult sheep and 3.4% of lambs were infected on meat inspection, with prevalence levels in the adult population as high as 61%, particularly in western Australia. Subsequent studies have reported a steady decline in prevalence. The introduction of a CLA vaccination in 1983 and its growing popularity in the farming community are largely responsible for this. CLA lesions were discovered in 7.1% of killed sheep and 0.64% of lambs in a 1986–1987 slaughterhouse investigation carried out by meat specialists in New Zealand (Abdolmaleki et al. 2019). Studies in the USA, particularly in western regions, have shown an average prevalence of disease among sheep as high as 42.5%. Similarly, other studies in the Canadian province of Quebec have found clinical CLA prevalence among adult slaughtered sheep ranging from 21% to 36%.

In many nations, C. pseudotuberculosis is rather prevalent, while

incidence rates vary. While the disease's seroprevalence is lower in other nations, which lessens the possibility of strains spreading, up to 75% of sheep in Brazil may be afflicted. According to Correa et al. (2018), 21% of sheep transported to slaughterhouses in Canada had been infected, compared to 26% of sheep in Australia. In the United States, Texas and California are the endemic regions for the disease in horses. The annual disease prevalence in these areas is 5–10%, but large increases and sporadic outbreaks have occurred. In 2002 and 2003, thousands of horses were reported to have been infected with C. pseudotuberculosis in Wyoming, Utah, Kentucky, and Colorado, where the prevalence of the disease has historically been low. Small outbreaks of 350 symptomatic cases were also reported in Alberta, Canada, in 2013, and in British Columbia in 2010. According to studies conducted in Albertan slaughterhouses, 8% of all corpses are killed to remove CLA lesions, and up to 5% of sheep and 0.03% of lamb carcasses are rejected for CLA (Farias et al. 2018). Studies on Brazilian sheep and goat farms, particularly in the southeast regions of Minas Gerais, have shown a notable prevalence of the disease, with an estimated frequency of 70.9% in sheep and 78.9% in goats. CLA has been identified as the main contributor of sheep carcass abnormalities in South African slaughterhouses (Babacan, 2024). CLA was responsible for losses ranging from 0.24% to 0.3% of all sheep carcasses, with trimming unwanted meat from the carcass resulting in significant additional losses (Li et al. 2018). A survey of small ruminant farms in Malaysia revealed an average illness prevalence of about 30% due to Caseous Lymphadenitis (CLA). Three clinical forms of C. pseudotuberculosis were found in 827 dairy cattle in an Israeli study conducted between 1989 and 2001. The more prevalent form caused skin ulcer abscesses and led to the removal of 16.3% of diseased animals from the herd. According to Oreiby et al. (2014), other types of the disease have invaded visceral lymph nodes and caused mastitis in cattle.

The enzyme phospholipase D is the main factor that contributes to the pathogen's capacity to infect animals. This enzyme is classified as an exotoxin since it is a toxin that is released outside of the bacterial cell (Magdy et al. 2022). The infection can move to secondary sites when phospholipase D destroys the connections in the host cell membranes. These exotoxins are consumed by phagocytes, which then carry them to local lymph nodes, where they result in the disease's distinctive lesion (Figure 3). Since phospholipase D breaks down the phospholipids that comprise the toxin-containing compartment inside the macrophage, it is not broken down during transport. These phospholipids are also found in the macrophage's cell membrane, which will ultimately cause the cell to die (Osman et al. 2018).

Clinical and Pathological Characteristics of Sheep Caseous Lymphadenitis

C. pseudotuberculosis, the term "caseous lymphadenitis" refers to the traditional association of infections in sheep with the development of pyogranulomas (Oreiby et al. 2013). The two primary types of lesions are visceral and external, and they can coexist in the same animal. Abscessation of the externally palpable lymph nodes is a characteristic of the external type. Depending on the organism's initial place of entry, any of the body's superficial lymph nodes could be impacted. Less frequently, the subcutaneous tissues may develop isolated purulent lesions that are not immediately connected to the surface lymph nodes. According to Radostits et al. (2000), these lesions can manifest as structured abscesses, characterized by swelling, fibrous encapsulation, loss of covering hair, and final rupture that releases pus. Abscesses in the internal lymph nodes and other organs are linked to the visceral kind. In sheep, the lung parenchyma and mediastinal lymph nodes are the main sites of these internal CLA lesions. The liver, kidneys, or udder may also sustain lesions; less frequently, the heart, testis, scrotum, uterus, joints, brain, or spinal cord are harmed (Baird and Fontaine, 2007).

Sheep Pathogenesis

The bacteria quickly spread to the local drainage lymph node after first entering. Numerous little pyogranulomas grow in size and combine to generate bigger abscesses in this location. Occasionally, the infection spreads further through the blood or lymphatic system, resulting in lesions that resemble those in other organs. Chronic and often lifelong illness is the norm rather than the exception due to the nature of these slowly progressing CLA lesions. Years after the initial infection, abscesses may still harbor live germs. After a long period of apparent quiescence, the disease may also reactivate, resulting in the formation of lesions at new locations (Paton et al. 2003).

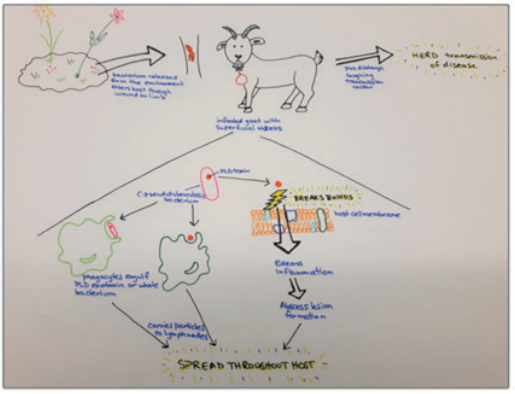

Figure 3. *Corynebacterium pseudotuberculosis pathogenesis*

Source: https://mechpath.com/2017/12/13/corynebacterium-pseudotuberculosis/

Sheep experimentally infected with CLA have been successfully infected by intradermal, intratracheal, subcutaneous, intravaginal, intravenous, and intralymphatic injection. (Fontaine et al., 2006; Nagy, 1976). However, it is believed that spontaneous infections primarily enter through the skin (Baird & Fontaine, 2007). This early infection is facilitated by minor cutaneous cuts and abrasions, especially those caused by shearing (Ali et al., 2016). Other hypothesized intermittent entry routes include castration or docking wounds and the umbilicus in neonatal animals. The many head and neck lesions observed in goats and the few observed in sheep from the Antipodes and North America have been linked to entry through the oral cavity. (Paton and others, 2003). However, it is believed that the more distant parts of the digestive system do not work as a portal of admission for the organism, even in cases where parasite damage is present (Domenis et al., 2018).

A broth culture of C. pseudotuberculosis injected intratracheally may cause dispersed pulmonary abscesses, according to Brown and Olander (1987). However, some studies have indicated that a systemic infection that originates in another area of the body may cause similar pulmonary abnormalities. The majority of internal lesions after intravenous inoculation of lambs with C. pseudotuberculosis were thus found in the lungs and associated thoracic lymph nodes (Ali et al., 2016). Furthermore, it has been shown that in natural ovine CLA infections, the distribution patterns of pulmonary lesions are more consistent with hemogenous or lymphogenous transmission than with aerogenous dissemination (Paton et al., 2003). Therefore, it would seem that even while there is a theoretical danger of infection entering through the respiratory system, it is not very significant.

Human instances

Human infections with C. pseudotuberculosis are rare, although most instances that have been reported have been connected to occupational contact; one case was discovered in 1988 and included the ingestion of raw goat meat and cow milk (Yaacob et al., 2021). There have been about 25 documented occurrences of this microbe infecting humans in the literature (Liu et al., 2005). In their assessment of 22 instances, Peel et al. (1997) found that infected persons typically symptoms, lymphadenitis, and abscesses. A youngster who had come into contact with polluted farm animals had suppurative granulomatous lymphadenitis, according to Mills et al. (1997). An ocular implant-related C. pseudotuberculosis infection in a patient's eye was documented by Liu et al. (2005). In the majority of instances, the afflicted lymph nodes were surgically excised and the patients were treated with antibiotics (Liu et al., 2005).

C. Pseudotuberculosis Infection General Aspects

The bacteria C. pseudotuberculosis was first discovered to be the cause of caseous lymphadenitis (CLA) in sheep and goats. However, it has since been isolated from other species, such as horses, where it causes ulcerative lymphangitis in cattle, buffaloes, swine, camels, and humans, as well as pigeon fever (Dorella et al., 2006). The severity of CLA is expected to be correlated with the pathogenic bacterium's ability to form biofilms. Many diseases are caused by a complex and organized microbial community called biofilm, which has a tendency to stick to both living and inert surfaces in the presence of extracellular polymeric substances (EPS) (Yahya et al., 2018). Because the bacterial community in the biofilm exhibits different gene and protein expression patterns from its planktonic counterpart, which usually leads to different metabolic and antimicrobial resistance profiles, the bacteria within the biofilm become more resistant to antimicrobial treatment (Giaouris et al., 2013). According to the National Institutes of Health (NIH), 65% of all microbiological and 80% of chronic disease are associated with biofilm formation (Yaacob et al., 2021).

Diagnosis

In order to successfully control CLA, infected animals must first be identified so that they cannot come into contact with uninfected animals. The detection and culture of C. pseudotuberculosis is the diagnostic criterion for CLA (Domenis et al., 2018). Even while there may not be many viable bacteria in chronic abscesses and occasionally sterile lesions are found, it is usually possible to extract the organism from lesions of all ages. Abscesses in sheep's exterior lymph nodes are a strong indicator of the illness, especially if multiple animals in a group have the same condition (Baird & Fontaine, 2007). Suppurative lymphadenopathy can also result from other bacterial infections, including Arcanobacterium pyogenes, Actinobacillus licheniformis, and in certain nations, Staphylococcus aureus subsp. anaerobius. These diseases are typically intermittent and are rarely considered a flock problem (Liu et al., 2005).

Many serodiagnostic assays have been developed to address the problem of clinically detecting CLA; however, most of them lack either sensitivity or specificity (Ali et al., 2016). Nonetheless, eradication and control efforts benefit from a number of diagnostic methods based on the enzyme-linked immunosorbent test (ELISA) (Dorella et al., 2006). Recently, ELISA techniques have been developed to detect gamma interferon (IFN-γ), a marker of cell-mediated immunity against C. pseudotuberculosis. The IFN-γ ELISA test appears to be more sensitive than the conventional antibody ELISA in detecting previous infections in goats, and it appears that vaccination in sheep has no effect on it (Yahya et al., 2018). Another novel method that shows

promise for the diagnosis of CLA is the use of polymerase chain reaction (PCR) testing specific to C. pseudotuberculosis to identify bacteria isolated from abscesses (Çetinkaya et al., 2002).

C. pseudotuberculosis cultivated from clinical samples can be recognized in the lab based on its enzyme profile and capacity to use different sources of carbohydrates. The advent of tiny, patented, standardized test kits, such the Analytical Profile Index (API) identification system (bioMérieux [UK], Basingstoke, Hampshire, UK), has significantly streamlined the biochemical profiling process. The "API Coryne" kit, which measures enzymatic activity or carbohydrate fermentation and is used to identify coryneform bacteria, comes with 21 different test substrates. Following inoculation with the test organism and incubation for a predetermined period of time, the metabolic byproducts of the enzymatic tests result in the development of specific color changes, either naturally or following the addition of reagents. Substrate fermentation testing also uses colorimetric measurement of pH change. Following that, a numerical profile is generated using a particular combination of positive and negative test results, and the database is "interrogated" using proprietary software (Yahya et al., 2018).

Control

Because of the tight capsule that surrounds them, live bacteria remain protected inside abscesses, making it difficult to control CLA with antibiotics (Williamson, 2001). It is widely accepted that immunizing healthy animals and identifying and removing affected animals are the best ways to control the disease. However, such an approach may be hampered by the challenges of early clinical identification of sick animals.

Vaccines

The majority of marketed caseous lymphadenitis vaccinations on the market today combine protection against additional infections. Among these are Clostridium novyi, Clostridium chauvoei, Clostridium tetani, Clostridium perfringens, and Clostridium septicum (Paton et al., 2003). Inactivated phospholipase D (PLD) is the basis for these vaccines, also referred to as toxoid vaccines. Paton et al. (2003) evaluated the effectiveness of a mixed toxoid vaccine against CLA and discovered that it hindered the disease's transmission throughout the flock and reduced the number and size of CLA lung abscesses. Goats cannot be vaccinated with all of the vaccinations that are approved for use in sheep. Furthermore, revaccination is advised for goats at six-month intervals, but the recommended vaccination schedule for sheep includes two priming doses for lambs and annual boosters for adult sheep (Dorella et al., 2006).

Treatment

A variety of antibiotic compounds can affect C. pseudotuberculosis in vitro. The most widely used antibiotic classes have been demonstrated to inhibit bacterial growth and multiplication in routine laboratory-based assays (Abdolmaleki et al., 2019). The thick encapsulation surrounding the usual lesions and the thick, caseous structure of the pus inside make clinical CLA often resistant to antibiotic therapy in vivo. It is also thought that the organism's intracellular nature during various stages of the illness cycle provides some defence against several regularly used antibiotics. As an alternative to culling, surgical treatment of external lesions has been suggested for particularly significant animals. To lower the risk of recurrence, parenteral antibiotic treatment for 4–6 weeks has been advised, regardless of whether the lesion is surgically removed or is just lanced and washed up every day until it heals (Abdulrahman et al., 2020).

An article claimed that a combination of oxytetracycline and rifamycin could successfully treat sheep infected with C. pseudotuberculosis (Senturk & Temizel, 2006). Rifamycin has a proven ability to destroy vulnerable intracellular bacteria and is mostly used to treat Mycobacterium leprae and Mycobacterium tuberculosis infections. In this brief study, ten rats with CLA received twice-daily rifamycin treatment for ten days. Additionally, patients received injections of a depot formulation of oxytetracycline at three-day intervals. This led to what was referred to be a clinical remission of the CLA lesions, which decreased in size in the affected sheep's external lymph nodes (Baird, 2007). The report did not specify how long the sheep were disease-free, and no necropsies were performed after the rigorous treatment regimen. Before treating CLA instances becomes a practical substitute for culling, more research is needed.

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Chapter 4

PARTRIDGE FARMING

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Partridge; A medium-sized bird with plumage of various colors, a thick body, and a short tail, belonging to the Galliformes order, within the Phasianidae family of the Phasianinae suborder, and classified under the genera Alectoris and Perdrix. (Çetin and Kırıkçı 2000, Turan 1990, Özçelik 1995). The most common partridge species in Turkey is the red-legged partridge, which is the most well-known and beloved of wild birds. (Çetin ve ark 1997, Çetin ve Kırıkçı 2000).

Distribution of partridge populations in our country;

Ø Chukar partridge (Alectoris chukar); In the high elevations of the Taurus Mountains, except for the coastal areas of Central and Eastern Anatolia and the Mediterranean region,

Ø Rock partridge (Alectoris graeca); In Western Anatolia, Thrace, Marmara, and the Aegean region,

Ø Caspian partridge (Tetragallus caspius); in the northern regions of Eastern Black Sea and Eastern Anatolia and in the high and rugged parts of the Taurus Mountains,

Ø Grey partridge (Perdix perdix); in Thrace, Marmara, Inner Aegean, Central Anatolia, and Eastern Anatolia,

Ø See-see Partridge (Jet Partridge) (Ammoperdix griseogularis); reported to be found in the eastern region, the valleys irrigated by the Euphrates and Tigris rivers, and the slopes of these valleys (Boyla 1995, Erencin 1977, Robbins 1998, Turan 1982).

Objectives of Partridge Breeding:

· To offer consumers alternative products with different tastes and aromas by producing different types of poultry meat and eggs,

· To support the preservation of natural balance and life by breeding and releasing them into nature or hunting grounds,

· To generate income for the country's economy by increasing tourism revenues through hunting tourism,

· For hobby purposes,

· To contribute to the creation of a new line of business and thus create employment.

Partridges play an important role in preserving the natural balance by eating worms, insects, and even harmful weeds that damage crops such as wheat, barley, and oats cultivated by humans. However, their numbers have rapidly declined due to uncontrolled and uninformed hunting, pesticides used for agricultural control, and chemical fertilizers (Çetin and Kırıkçı 2000, Kuyulu 1948).

Important considerations in partridge breeding:

The region, climate conditions, and other factors are extremely important when aiming for large-scale production of different partridge species. These conditions include:

· The ability of the selected species to survive in the chosen region,

· The creation of suitable conditions for reproduction or artificial reproduction,

· A hatchery containing compartments where eggs can be stored and disinfected, along with an incubator and all hatching equipment,

· Special rearing compartments or cages for partridge chicks,

· Walking areas where partridges can meet their natural needs (roosting, dust bathing, etc.) when not in production,

· Sufficient space and other requirements to enable expansion of the operation when necessary,

· Paying attention to health, safety, and hygiene rules during production,

· Taking measures to increase productivity in pre-development and hatching compartments during incubation.

Failure to perform proper disinfection during the incubation period causes deaths in the first days due to salmonella and coli, especially in chicks obtained from partridge eggs raised in open areas (Dumanlı and Özer 1985).

Egg Laying, Egg Production, and Egg Weight in Partridges

Partridges begin laying eggs at approximately 30–32 weeks of age (8 months) and in the spring in the wild (Woodard et al. 1993). In the wild, a partridge lays and incubates an average of 15 eggs. Intensively reared partridges generally lay eggs in late March or early April, and egg laying continues until mid-August. The egg-laying period lasts approximately 16–20 weeks. Çetin et al. (1997) reported this period as 68–83 days for chukar

partridges, Yannakopoulos (1992) reported it as 120 days for rock partridges, and Aysöndü (2005) reported it as 124 days for rock partridges. Kırıkçı et al. (1999) determined the egg-laying period as 132 and 118 days in groups subjected to artificial and natural lighting, respectively. Çetin et al. (2002) determined the egg-laying period as 94 and 127 days for first- and second-instar rock partridges, and Kırıkçı et al. (2003) determined the egg-laying period as 113 and 123 days for first- and second-instar rock partridges, respectively.

Partridge eggs can be used for food and breeding. Hatching eggs should be of normal size. The chance of hatching chicks from small or overly large eggs is low, and using undersized eggs is risky because this undesirable trait can be passed on to future generations through inheritance, resulting in smaller eggs (Esen 1998). Large eggs generally have lower hatchability. Double-yolked eggs, on the other hand, do not hatch (Aksoy 1994, Esen 1998). Partridge eggs weigh 16–25 g, are light milky brown, and have brown spots. Partridge egg weights have been reported to range from 19.16 to 22.50 g (Song et al. 2000, Woodard et al. 1982). If we consider this situation according to partridge species:

Ø In chukar partridges (Alectoris chukar), egg weights range from 18.99 g to 22.50 g (Alkan and Türker 2021, Alkan et al. 2007, Aygün and Olgun 2019, Çağlayan et al. 2014, Çetin et al. 1997, Karabağ et al. 2010, Kırıkçı et al. 1999, Kırıkçı et al. 2018b, Woodard et al. 1982, Yannakopoulos 1992).

Ø In rock partridges (Alectoris graeca), egg weights range from 17 to 23.86 g (Aysöndü and Özbey 2008, Çağlayan et al. 2009, Kırıkçı et al. 1999, Kırıkçı et al. 2004, Özbey and Esen 2007).

Ø In red-footed partridges (Alectoris rufa), egg weights are reported to range from 19.63 to 19.80 g (Gomez-de-Travecedo et al. 2014a, Gómez-de-Travecedo et al. 2014b, González Redondo and Martínez-Domínguez 2019).

Partridge Shelters and Breeding Systems

Adult partridges are highly resistant to heat and cold. Therefore, semi-open shelters built in open areas are very suitable for partridge breeding. Shelters should be constructed to protect against direct sunlight and wind (Embury 1997, Kırıkçı et al. 1999).

Partridge shelters are more suitable if they are built with enclosed areas, exercise areas, and areas that open to the outside environment. Partridges can be successfully raised in large flocks if the area is suitable. A coop with an exercise area measuring 6 m long, 1.2 m wide, and 1.2 m high, and an enclosed area measuring 1.2x1.2x12 m, is sufficient for 50 partridges. If more animals are placed in the coop, care and feeding may become more difficult, and cannibalism may occur more frequently (Çetin and Kırıkçı 1998, Kırıkçı et al. 1999).

Controlled breeding is crucial during the early stages of partridge chicks (especially the first 4 weeks). If the necessary care is taken during this period, losses will be minimized and successful rearing will be achieved. Although partridges are quite resilient to adverse rearing conditions in other periods, the semi-open rearing system is the most suitable rearing system. Other systems (closed or open) can be used comfortably for partridge rearing.

Care and Feeding of Partridge Breeding

Partridges have two distinct periods during their adult years:

Ø The non-laying period,

Ø The laying or egg-laying or egg-laying period.

Outside of the laying period, the birds' energy needs are extremely low. This is because feather development is complete, a significant portion of physiological functions are not performed, and the bird's weight is close to its adult body weight. Furthermore, intensive feeding during this period can cause the birds to gain fat, leading to negative consequences such as a decrease in fertility rates during the laying period. Therefore, the energy content of feed during this period should not exceed 2500 kcal ME/kg and crude protein should not exceed 22% (Başer and Küçükyılmaz 2000, Kırıkçı and Çetin 1999).

Nutrient needs in the birds increase during the laying period with the onset of production. Increases in energy, protein, and mineral requirements, especially calcium, occur. Nutrition during this period is also important due to the increased hatchability and fertility rates of eggs. Providing these feeds 1-2 weeks before the start of egg-laying is particularly important for maintaining consistent shell quality and high breakage resistance. Magnesium and zinc are also of particular importance in the nutrition of partridges. They are used to create the sheen on the feathers (Kırıkçı and Çetin 1999).

Although partridges live in pairs in nature during the breeding season, the male:female ratio in intensive farming is 1:3 (can be increased to 1:5) (Çetin et al. 1997, Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999). Selecting the breeding flock in late winter positively affects fertility before egg-laying begins. The first egg is taken at 32 weeks of age. In the selection of breeding partridges: Care should be taken to ensure that their live weight is not low (e.g., 450 g for males and 400 g for females) and that male partridges, in particular, have long and strong spurs (Beani et al. 1993, Çetin et al. 1997, Yannakopoulos 1992). Partridges in this period should be fed a ration containing 24% HP, 2850 kcal ME/kg (Table 2) (Çetin et al. 1997, Monetti et al. 1998, Monetti et al. 1990, Meyer and Milliam 1986, Hermes et al. 1984b). In addition to this ration, green forages such as alfalfa can be given (Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999).

Artificial lighting should be provided to male partridges starting two weeks before mating. Partridges lay eggs from early spring to mid-summer under natural conditions. This production season can be extended with 16 hours of artificial lighting at a maximum intensity of 10 lux/m^2 (Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999, Yannakopoulos 1992).

If breeding is carried out in closed coops and artificial lighting is used, fertile eggs should be expected 2-3 weeks after mating begins. However, in flocks using natural lighting, the first eggs obtained are fertilized (Çetin et al. 1997, Çetin and Kırıkçı 2000).

Partridges should preferably be used for breeding for 1 year. However, in flocks that start with a small number of partridges, there is no harm in using the breeders for 2 years (Çetin and Kırıkçı 2000).

Incubation of Partridge Eggs

With the start of the laying period, shelters must be kept as clean as possible to increase the number of eggs available for incubation. Partridge eggs should be collected at least twice a day. This is because partridges raised under intensive conditions frequently exhibit behavior that leads to egg breaking, which can increase the rate of broken eggs. Furthermore, in very hot weather, eggs must be collected more frequently to prevent embryonic development (Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999, Kırıkçı et al. 1999). Regardless of how low the bacterial count on the eggs is, it tends to multiply rapidly, making fumigation of the eggs important. Potassium permanganate and a 37% formaldehyde solution are used in the fumigation process. The amount to be used is 20 g of potassium permanganate and 40 ml of formaldehyde solution per 1 m^3 of machine or cabinet volume. Furthermore, the hygiene of the incubator can be ensured by using a cheap and safe disinfectant. For example, washing the machine surfaces with a mixture of 0.5 kg of soda and 18 liters of water is very suitable. The ceiling of the machine should first be washed with water and then with disinfected water (Çetin and Kırıkçı 2000, Embury 1997, Kırıkçı and Çetin 1999, Kırıkçı et al. 1999, Woodard and Morzenti 1975).

If an incubator suitable for other birds is to be used, trays suitable for the smallest partridge eggs (up to 17 g) are required (Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999). Embury (1997) reported that hatching efficiency is lower when eggs are incubated after being stored for more than 14 days, while Hermes (1991) reported no significant change in hatching efficiency within a 4-week storage period. The temperatures of the collection rooms are provided in Table 1 based on the storage period.

Table 1: Storage conditions for breeding eggs (Aydoğan, 1998)

Storage Period	Maximum Storage Temperature	Humidity Level
1-3 days	20 ^{0}C	% 75-85
3-7 days	13-16 ^{0}C	
Over 7 days	11-12 ^{0}C	

Properly collected and stored partridge eggs have an average incubation period of 24 days (21 days for pre-development and 3 days for hatching). Eggs stored longer than 14 days exhibit reduced hatchability (Çetin and Kırıkçı 2000, Embury 1997, Kırıkçı and Çetin 1999, Kırıkçı et al. 1999, Woodard and Morzenti 1975). In natural incubation, the turning of the broodstock with their feet and wings is done manually or automatically in machines. This eliminates any temperature differences between the top and bottom of the broodstock. This prevents the membranes from sticking to each other and the brood from sticking to the membranes. The brood is then allowed to settle on its axis. Inadequate turning can lead to problems such as embryo mortality, reduced hatchability, and reduced viability. Eggs are placed in the machine with their pointed ends down or on their sides. Incubated eggs are turned at least three times a day. More frequent turning has a positive effect on efficiency. Turning is stopped on day 21, and the eggs are transferred to the hatching section. The temperature in the incubator should be 37.6°C and humidity 60%; at hatching, the temperature should be 36.5°C and humidity 70%. If the relative humidity in the incubator is too low, embryo mortality is high. If it is too high, the chicks hatch with stickiness or suffocation under the shell occurs (Embury 1997, Woodard and Morzenti 1975).

When hatching occurs late or early, the following issues are considered:

Ø Leaving the machine on for extended periods other than the regular on and off times.

Ø Differences in the storage times of hatching eggs.

Ø Improper temperature control of the machine.

Ø Prolonged power outages.

Ø Differences in the waiting time between eggs, parent age, storage method, and egg weight.

Care of Partridge Chicks

It is ideal to keep chicks in wire cages with mesh that will not harm their feet. However, rooms heated to 33°C with wood shavings as bedding can be used for rearing. The temperature in the main machine or rooms is reduced by 1°C every 3 days and stabilized at 21°C. Quail chicks should be heated for

4-5 weeks depending on weather conditions (Çetin and Kırıkçı 2000, Kırıkçı and Çetin 1999).

Chicks taken from the incubator should be fed 3-4 hours after being given 5% sugar water. Depending on the rate of development observed in partridges from the first week onwards, they require not only high protein but also high energy levels. Furthermore, these animals require a large amount of energy not only for growth but also due to their active nature and high feather development. During this period, the protein level in feed should be 27-28% and the energy level should be 3000 Kcal ME/kg. The depth of the waterers provided for chicks to drink from should be sufficient to prevent drowning and wetting (Çetin et al. 1997, Embury 1997, Hermes et al. 1984a, Hermes et al. 1984b).

Some Productivity Performance of Partridges

The productivity performance achieved in partridges is given in Table 2 and Table 3.

Table 2: Egg Production and Incubation Performance of Partridges

Egg Production (Number)	Fertility Rate (%)	Hatching Rate (%)	Hatchability Rate (%)	Source
53.36	88.05	85.31	88.04	Yannakopoulos 1992
11.20-38.40	57.14-89.06	53.57-81.25	91.11-93.75	Çetin et al. 1997
21.27-67.10	74-90	61.5-77.8	----	Woodard et al. 1981
44.38-57.88	81.82-90.64	75.92-87.96	92.79-97.05	Kırıkçı et al. 1999
34.16-45.65	93.48-96.90	77.11-78.47	80.97-82.49	Çetin et al. 2002
---	---	60.2-65.3	88.04	Woodard ve Morzenti 1975
---	96.9-82.10	78.47-65.68	80.97-80	Çetin and Kırıkçı 2001 (Male: Female Ratio =1:3 ve 1:4)
---	94.20-81.40-66.60	85.70-74.30-58.80	91-91.20-88.30	Kırıkçı et al. 2018a (May, June, July)
30.28-30.59	81.53-92.22-	63.78-70.98	76.70-78.78	Kırıkçı et al. 2006
---	87.08-88.58	72.56-74.07	83.42-83.35	Çetin et al. 2008 (Morning-Afternoon)
51.55-42,80	83.34-75.73	66.89-59.80	80.18-80.22	Kırıkçı et al. 2003 (1. ve 2. period)
---	88.32-77.92	78.97- 63.20	89.42- 81.11	Çağlayan et al. 2014 (Spotted-Unspotted)
49.35-49.43	90.25- 72.55	78.20- 67.09	86.64-92.48	Aysöndü and Özbey 2008 (Cage-Ground System)
---	83.47- 77.08-69.92 ve 68.01	72.97- 57.13-59.31 ve 58.68.	87.68- 74.43-84.40 ve 85.79	Alkan et al. 2008

---	66.0-76.0	48.0- 62.0	81.6-87.9	González-Redondo and Martínez-Domínguez. 2019 (Red-legged partridge)
	95.33	89.33	93.71	Bilgiç 2022

Table 3: Partridge Feed Performance (LW=Live Weight, FCR=Feed Conversion Ratio)

Feeding Period (Weeks)	Final Body Live weight (g)	Feed Intake (g)	feed efficiency ratio	Carcass Yield	Source
18	600	3600	6.00	---	Embury 1997
24	445.86	---	---	---	Çetin et al. 1997
20	595-630	---	---	---	Woodard et al. 1981
12	425.50	1582.27	3.84	71.79	Kırıkçı et al. 1999
20	468	4658.20	9.89	---	Kırıkçı et al. 1999

Table 4: Embryonic Death Rates (%)

Early Embryonic Death Rate	Mid Embryonic Death Rate	Late Embryonic Death Rate	Total	Source
1.68-1.00	4.30-2.32	7.37-4.18	13.35-7.50	Aysöndü 2005 (Cage-Ground System)
1.27-0.99	2.32-1.42	4.03- 4.01	7.62- 6.42	Çetin et al. (2008) (Morning-Afternoon)
1.75	1.22	3.32	6.29	Bilgiç 2022
			6.29-15.41	Alkan et al. 2008
			10.59-18.89	Çağlayan et al. 2014 (Spotted-Unspotted)
			8.40-7.10-7.80	Kırıkçı et al. 2018a (May, June, July)

As shown in Table 2, under normal care and feeding conditions, 40-70 eggs per bird can be obtained annually from partridges. Due to the high incubation efficiency and hatch rate, it appears possible to obtain 35-40 chicks per partridge in a year.

Although partridge farming is generally done for hunting tourism purposes, they can also be raised for meat production due to their delicious meat and very low cholesterol levels in their carcasses. They can also fetch higher prices than other poultry species due to their rarity and deliciousness (Çetin and Kırıkçı 2000, Günlü et al. 2001).

Diseases and Disease Prevention

Partridges are animals that are quite resistant to infectious diseases and hot and cold weather conditions compared to other poultry species. An important disease seen in partridges that causes significant losses is coccidiosis. It is particularly common in newly hatched chicks, and once infection occurs, treatment is very difficult. Adding coccidiostatic drugs to water and feed is very useful for prevention (Kırıkçı and Çetin 1999, Ruff and Wilkins 1990).

Another of the most common diseases seen in partridges is cannibalism. Cannibalism is controlled by beak trimming at 0-3 days of age, and this procedure may be required every 6 weeks. Rearing in large, open areas, providing an appropriate ration and green feed, reducing light intensity, and maintaining high humidity can also help prevent cannibalism (Kırıkçı and Çetin 1999, Embury 1997). In addition, monthly internal and external parasite examinations should be performed.

Releasing Partridges into Hunting Grounds

One of the most important objectives of partridge breeding is to release them into hunting grounds for hunting. This contributes to the development of hunting sports and hunting tourism, as well as helping people escape the tedious city life and monotony of today, and maintaining their physical and mental health (Çetin and Kırıkçı 2000).

Hunting grounds should be selected in mountainous, rocky, and forested areas where agriculture is not practiced and human population is sparse. These areas should have sufficient water. Hunting grounds should be established 5-10 km away from agricultural areas to avoid the damage that agricultural pesticides and fertilizers can cause to partridges. There are many unused areas in Anatolia that meet these criteria (Çetin and Kırıkçı 2000).

Conclusion

Considering that partridge farming has become an industry in countries across America and Europe and generates significant foreign exchange revenue for these countries due to its role in hunting tourism, it is unfortunate that Turkey has yet to develop this sector, despite its climate, geographical conditions, and tourism potential being highly suitable for partridge farming. Conducting the necessary studies to ensure that the number of game animals in our country reaches an adequate level is essential for the development of the sector. At the same time, bringing poaching under complete control will contribute both to the preservation of the natural balance and to the creation of new job opportunities.

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Chapter 5

AN OVERVIEW OF CELL SEPARATION TECHNIQUES

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1. INTRODUCTION

The diverse characteristics and complex structures of cells give rise to a wide range of scenarios in medical and biotechnological applications that require the isolation of specific cell types. This necessity has made cell separation techniques a significant topic, emphasizing their importance and facilitating the examination of the fundamental principles in this field.

"Cell isolation" is one of the terms used to express cell separation. In addition to this term, "cell separation", which is the equivalent of cell segregation, is preferred, as it provides a more specific explanation. Cell separation is the process of separating cells according to their different characteristics. These characteristics can include cell size, density, electrical charge, surface proteins, and gene expression. This separation process allows for the segregation of a group of cells by selecting them on the basis of their predetermined properties. Cell separation is generally used for various purposes, such as research, diagnosis, obtaining individuals of the desired sex and therapy. The importance of this process becomes even more significant in cell-based therapies, as successfully isolating a specific cell type has a direct effect on the effectiveness of treatment processes.

There are many methods used for cell separation. The choice of one of these methods for a particular purpose depends on how the cell is obtained, the structural characteristics of the tissue, and the methods used to identify the cell type. Among the techniques applied for cell separation, many include density gradient centrifugation**,** magnetic separation, ultrasonic separation, and fluorescence-activated cell sorting (FACS). These techniques help separate cells on the basis of specific characteristics and collect separated cells.

This chapter focuses on objective and automated systems used for cell type determination, provides a foundation for cell separation, and addresses the advantages and disadvantages of different techniques. Additionally, the importance of the identification process required for cell separation is emphasized.

2. CELL SEPARATION TECHNIQUES

The main goal of cell separation is the isolation of a viable single cell type from a heterogeneous cell population for various purposes, such as diagnosis, treatment, or research. This becomes even more important in cell-based therapies. In cell separation, it is necessary to first determine the type of the desired cell, that is, its identification, and then separate and transfer it to another environment. Although the term "cell isolation" is also used to refer to cell separation, it is more appropriate to use the term "cell separation."

Positive and negative separations are the basic principles in cell separation. The aim of positive separation is to isolate target cells from the

cell population, whereas negative separation aims to reduce the number of other cells in the cell population except for the target cells. Both methods have advantages and disadvantages. In positive separation, due to the use of highly specific antibodies, the purity of the obtained target cell population is greater. In negative separation, as multiple different antibodies are used, the process is more complex and less efficient. While the purity level can be further increased with subsequent processes in positive separation, this is not possible in negative separation. However, if the cells obtained by positive separation may contain antibodies or labeling agents that may negatively affect subsequent culture processes, the negative separation method may be preferred (Tomlinson et al., 2013; Hoeve et al., 2016; Rodrigues et al., 2016; Rahmanian et al., 2017).

Many methods can be applied for cell separation. The selection of the method to be applied is closely dependent on whether the cell will be obtained alive, the structural characteristics of the tissue from which the cell will be obtained, and the method to be applied in typing the cell to be separated. To separate a specific cell type, it must first be identified in a way that objective and automatic systems can recognize; that is, it must be typed. For this purpose, it is necessary to define one or more stable characteristics that are specifically expressed by the cell type in question within the cell population and will help distinguish it. These characteristics may include cell shape, cell size, membrane molecules, gene pattern, amount of DNA, surface static electric charge, speed of movement in liquid medium, or a feature to be defined in the future (Table 1.).

Cell physiology, histological staining properties, other visual findings, selective cell culture medium, cell redox potential, and cell behaviors are characteristics utilized in cell separation (Table 1). Antibodies that can bind specifically to specific molecules on the cell surface, also called surface antigens, are also used to selectively separate cells. These antibodies that specifically bind to cells are labeled with fluorochromes or magnetic nanoparticles, which are utilized in the separation of labeled cells. The surface electric charge of the cell enables it to adhere to plastic or other polymer surfaces, thus allowing the separation of desired cell types in a suspension or free-floating cell population. Cell size and density enable the separation of cells in large quantities through precipitation, filtration, or density gradient centrifugation techniques.

The method to be applied in cell separation is selected on the basis of the following characteristics:

- Usable properties of cells for cell separation,
- Resistance of cell type-defining properties and viability to stress that may occur during the application of the method;

- Purity degree and quantity of cells to be obtained,
- Acceptable level of contamination, which should be zero if cell culture methods are to be applied;
- Suitable for the negative or positive separation method,
- Meeting the specific requirements of subsequent processes such as cell culture and nucleic acid/protein extraction;
- Time, laboratory needs, consumables to be used, and automation costs,
- Which antibodies or their single-chain fragments (aptamers) are used for labeling is among the most important factors.

Table 1. *Principles of basic cell separation techniques, positive/negative (desired/undesired) cell separation, purity levels and quantities of the separated cell populations*

Technique	Principle	Positive/ Negative	Purity	Yield
1. Density-based centrifugation	Cell density	Positive	Low	High
2.1. FACS	Surface antigen binding	Positive	High	Low
2.2. MACS	Surface antigen binding	Both positive and negative	High	Medium
2.3. Erythrocyte rosetting	Cell size + surface antigen	Both positive and negative	High	Medium
2.4. Aptamer binding	Surface antigen binding	Positive	High	Low
3. Filtration	Cell size	Positive	Low	High
4. Selective cell proliferation in culture	Physiology	Negative	Medium/ High	Low/ Medium
5. Laser-assisted microdissection cell separation (LCM = LMD); there is also a version using monoclonal antibodies	Morphology	Positive	High	Low
6. Raman spectroscopy	DNA amount	Positive	High	Adequate
7. Plastic/adhesion	Surface electric charge and adhesion	Male/female chicken embryo separation	High	High

2.1. Density Gradient Centrifugation

This technique enables the separation of specific cell types from a suspension layered over density gradient media. For this purpose, it is preferable to use a swinging-out rotor, which allows more precise layering. This approach

is particularly practical for the separation of blood cells. In this process, blood is layered onto special solutions of varying concentrations placed in conical-bottom tubes, which are then centrifuged at the recommended G-force. The desired cells form a layer just below the interface, whereas others sediment at the bottom. The cell layer of interest can then be collected via a pipette. Commercially available solutions of different densities are widely used for this purpose. Although this method is simple and inexpensive, it has limited specificity. Therefore, it is commonly used for the removal of dead cells prior to FACS, (magnetic-activated cell sorting (MACS), and single-cell separation procedures (Low & Wan Abas, 2015). Another technique, which is a modification of density gradient centrifugation, is cell separation, which utilizes the lifting force created by centrifugation. In the latest version of this technique, droplet-based samples that allow working with microliter volumes can be used (Phacilitate, 2022).

The density gradient media used for these purposes may be either discontinuous or continuous gradients. In the discontinuous gradients, distinct density bands are formed along the gradient, whereas in the continuous gradients, the density increases gradually without forming discrete bands. In discontinuous gradients, solutions of different concentrations are carefully layered within the tube. For the continuous gradients, the solutions with the lowest and highest densities formed a continuous gradient with increasing density along the conical test tube. Discontinuous density systems are widely applied in the separation of blood cells, whereas continuous density systems are more commonly used for the separation of bone marrow cells, Leydig cells, and intestinal epithelial cells.

Table 2. *Examples of commonly used media in density-based cell separation and some of their properties.*

Medium	Composition	Cell types separated
Lymphoprep	13.8% (w/v) sodium diatrizoate and 8.0% (w/v) polysaccharide	Mononuclear cells
Polymorphprep	9.1% (w/v) sodium diatrizoate and 5.7% (w/v) polysaccharide	Neutrophils
Polysucrose 400	A nonionic synthetic polymer of sucrose. Combined with sodium diatrizoate to create a density gradient.	A nonionic synthetic polymer of sucrose. Combined with sodium diatrizoate to create a density gradient.
Ficoll®	A nonionic synthetic polymer of sucrose. Combined with sodium diatrizoate to generate a density gradient. Compared with polysucrose, it has the advantage of being osmotically inert.	Leukocytes (WBCs) and erythrocytes (RBCs), mononuclear cells and granulocytes, viable and dead cells

Percoll®	A colloidal suspension of 15–30 nm diameter silica particles coated with polyvinylpyrrolidone (PVP).	All types of blood cells, splenic and lymph node mononuclear cells, lamina propria cells of the colon, microglia, hepatocytes, Leydig cells
Optiprep®	A 60% (w/v) aqueous solution of iodixanol	Alveolar cells, spermatozoa, gastric mucosal cells, hepatic stellate cells

The cytospin method is a long-established form of cell separation by centrifugation in which cells from samples such as blood, cerebrospinal fluid, peritoneal fluid, and synovial fluid are collected on the surface of a specially prepared base material, regardless of the cell type. The resulting cell smear can then be stained and examined. There are special centrifuges and systems developed for this purpose, where cells are collected on their surfaces. While the main advantage of the system is that the process can be carried out in a short time, the current system cannot separate specific cells, cell shapes are distorted, their viability is significantly reduced, and maintaining sterility is difficult (Bauer, 1987; Bauer & Hannig, 1988; Hengstschläger et al., 1997; Savaris, 1997; Bauer, 1999)

2.2. Immune Techniques

These techniques utilize monoclonal antibodies for cell typing and fluorescence or magnetic fields for cell separation.

2.2.1. Flow cytometry and fluorescence-activated cell sorting (FACS)

This technique uses fluorescence for cell typing. The system was developed to perform various measurements on individual cells rather than creating images. The system requires powerful laser optics and electronics combined with computer technology. For measurement, a cell suspension stained with fluorescent-labeled monoclonal antibodies or special fluorochromes (such as the DNA-specific stain Hoechst 33324 or fluorescein diacetate) that emit fluorescence when excited by an appropriate laser is introduced into the system. As the cell passes in front of the laser beam, fluorescence occurs if it is specifically stained. This fluorescence signal is captured by detectors and used in analyses.

The system can also be used for the separation of desired cell types with attached components. In this case, the system is called FACS, or fluorescence-activated cell sorter. To separate the labeled cell types, the principle of attraction of objects with different static electric charges is used. The cell type to be separated is deflected toward one of the metal plates charged with static electricity and collected in the lower tube. (Nunez, 2001; Ibrahim & Van Den Engh, 2007; Zu et al., 2009; Pathak & Banerjee, 2020). The most important application of FACS is the separation of spermatozoa carrying X

and Y chromosomes. The basic mechanism utilized in this process is that the X chromosome is larger than the Y chromosome and contains more DNA. When stained with a vital fluorochrome that specifically stains DNA, spermatozoa carrying the X chromosome have a relatively high fluorescence intensity and can be easily separated by FACS. Notably, antibodies are not used in this process (Otto et al., 1979; Pinkel et al., 1982; Garner et al., 1983).

2.2.2. Magnetic-Activated Cell Separation (MAGSEP/MACS) technique (Immunomagnetic separation, IMS)

In this technique, the cells to be separated are labeled with monoclonal antibodies via an immune technique, similar to flow cytometry. However, instead of fluorochromes, iron nanoparticles that become magnetized when exposed to a magnetic field are used to label the antibodies. The system's selectivity is quite satisfactory, it is relatively inexpensive, and it operates with little effort and cost. With simple isolation, $2x10^{10}$ cells can be purified in 10–45 minutes, and the target cells can be enriched 100-fold. In recent years, a column type of this system has also been developed (Tomlinson et al., 2013; Hoeve et al., 2016; Rahmanian et al., 2017).

2.2.3. Cell separation by erythrocyte rosette formation

Erythrocyte rosetting (E-rosetting) is spontaneous clustering formed by erythrocytes adhering to the membrane of a centrally located cell. It is accepted that human T lymphocytes are isolated from healthy individual's form E-rosettes (erythrocyte rosettes) with sheep erythrocytes. Rosettes were observed under a light microscope. In this process, the CD2 molecule on the T lymphocyte membrane binds to the sugar-based LFA-3 (lymphocyte function-associated molecule-3) homolog molecule on the sheep erythrocyte membrane (Onul, 1971; Wybran & Fudenberg, 1971; Armstrong & Cohen, 1999).

EA-rosette (Erythrocyte Antibody-rosette) is a rosette form created by antibody-coated erythrocytes with B lymphocytes that have Fc receptors for antibodies on their surface. Erythrocytes infected with malaria parasites, especially *Plasmodium falciparum*, also form E-rosettes with ABO group erythrocytes (Onul, 1971).

EAC rosette (Erythrocyte Antibody Complement-rosette) is a rosette form created by red blood cells in the presence of complement. The complement binds to the Fc region of the antibody. Finally, T lymphocytes with complement receptors bind to the complement, completing rosette formation.

If cell separation and subsequent processes are intended for purposes other than diagnosis or research, the rosette-forming cells are precipitated by an appropriate centrifugation process. This process includes density gradient centrifugation. Nonrosetting cells remain at the top. Depending on whether

negative or positive separation is intended, cells from the corresponding layer are used.

2.2.4. Cell separation by aptamer binding

Aptamers are peptide molecules or short-chain oligonucleic acids that can bind to specific target molecules. They are mostly in the structure of messenger ribonucleic acid (mRNA). These synthetically prepared molecules can be used for basic research and clinical purposes, as can macromolecular drugs. They are synthesized in the laboratory through the method of Systematic Evolution of Ligands by Exponential enrichment (SELEX).

Aptamers are divided into two main groups. These are DNA or RNA (ribonucleic acid) aptamers, which are generally oligonucleic, i.e., short nucleic acid chains, or peptide aptamers containing short variable peptide regions.

The synthesis of aptamers requires advanced technology. The initial DNA library is amplified via polymerase chain reaction (PCR), and its RNA transcript is created. This transcript is brought into contact with the target molecule to determine its specificity. If suitable, it is converted to complementary DNA (cDNA) by reverse transcription. This cDNA is also amplified via PCR and transcribed back into RNA. These processes are repeated 5–12 times. Since aptamers can adhere to small molecules, such as proteins, nucleic acids, cells, tissues, and organs, they have the potential to be widely used in biotechnology and therapeutic applications because of their ability to identify certain molecules (e.g., biomolecules and antibodies).

These synthetic molecules, which are flexible, are more compatible with the surface properties of target structures. With these properties, they better adhere to small or large target structures. They can also be used as alternatives to antibodies in research, diagnosis, and treatment as biosensors for binding to target structures. By conjugating gold nanoparticles or quantum dot dyes to aptamers, pointwise diagnosis can be performed (Röthlisberger & Hollenstein, 2018; Gray et al., 2020).

2.3. Cell Separation by Filtration

This cell separation method is based on the cell size. The cells smaller than the filter's pore diameter pass through the filter, while larger cells are collected on the filter. This method was first applied to separate tumor cells from smaller blood cells.

Some of the equipment developed for cell separation by filtration is passive devices that operate via gravity or capillary action, whereas others are active devices that work with vacuum pumps. The following table provides some characteristics of the commercial systems used (Table 3).

Table 3. *Filtration-based cell separation systems and their characteristics.*

Device	Pore size (µm)	Sample volume (ml)	Principle
Screencell Cyto®	7.5	3	Vacuum pump
Screencell MB®	6.5	3	Vacuum pump
Screencell CC®	6.5	6	Vacuum pump
ISET®	8	10	Vacuum pump
Metacell®	8	50	Capillary action

This system has advantages such as being simple, easy to use, and producing reproducible results; obtaining a high quantity and number of cells; and the ability to use the cells collected on the filter directly in subsequent processes. Its disadvantages include the low purity of the obtained cell population, loss of cells present in small numbers in the sample population, prevalence of false positives, and finally, the inability to use some cancer cells in subsequent processes owing to the loss of surface phenotypic characteristics during the procedure. The main significance of this method is that it provides hope for the possibility of isolating cancer cells from blood. To increase the sensitivity of the method, efforts are being made to combine it with techniques such as immune staining, cell examination systems, including microscopy, the fluorescence in situ hybridization (FISH) technique on filtered cells for chromosome analysis, and direct DNA and RNA analyses of cells captured on the filter (Arya et al., 2013).

2.4. Selective Cell Proliferation in Culture

In this method, certain cell types proliferate, while the remaining cell types are suppressed via cell culture media that promote the growth of the targeted cell type.

The basic principles of special cell/tissue culture media, also known as selective media, are prepared on the basis of certain cell characteristics. These characteristics include antibiotic resistance and the use of metabolic/biosynthetic enzymes. When antibiotic resistance is utilized, an antibiotic resistance gene is transferred to the targeted cells. Then, by adding antibiotics to the medium, resistant cells are allowed to survive. Viable cells are separated, and a cell population containing completely targeted cell types is obtained. The antibiotics used mainly for this purpose are bleomycin, puromycin, and hygromycin.

In this cell culture-based technique, which is mostly applied in hybridoma technology and uses metabolic/biosynthetic enzymes, a cell culture medium is used that ensures the expression of the gene encoding the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) enzyme only in the targeted cell type. This medium is also known as hypoxanthine-aminopterin-thymidine (HAT). The aminopterin in this medium blocks new DNA synthesis, whereas hypoxanthine and thymidine provide the raw material needed for the

alternative salvage pathway. The enzyme required for this alternative salvage pathway is HGPRT. Thus, only cells that can synthesize this enzyme survive in HAT medium. The enzymes used for this purpose include adenosine deaminase (ADA), aminoglycoside phosphotransferase (APH), dihydrofolate reductase (DHFR), histidinol dehydrogenase (hisD), thymidine kinase (TK), xanthine-guanine phosphoribosyltransferase (XGPRT), and cytosine deaminase (CDA). The application of this technique is relatively easy, the results are reproducible, and sufficient quantities of cells can be obtained. However, the technique has significant disadvantages, such as the risk of contamination, the presence of spontaneously resistant cell clones that do not carry the relevant gene, the high cost of some substances and special media used, and the time and labor required for both the proliferation and maintenance of the relevant cell clones (Aşan & Dağdeviren, 2012).

2.5. Laser-assisted Microdissection and Cell Separation

The system is based on marking the desired cell type or cell groups in appropriate tissue sections, which are stained or unstained, under a suitable microscope on a computer screen, and then dissecting this area with a laser. The dissected area is then removed from the tissue and transferred to an appropriate medium, either via a micromanipulator microscope with a suitable needle tip or with the help of laser pressure.

Unlike other cell separation techniques, this method can isolate even a single cell. As the procedure is performed under microscopic visualization, the entire process is under control. The micromanipulators used in this method began to be developed in the early 2000s. Recent developments have enabled the full automation of the process flow, including the recognition of the target cell.

Therefore, in the technique developed today, the identification of target cells can be performed by software algorithms as well as by the user on the microscope image. This technique allows for single-cell separation even in stained or unstained live tissue sections. While this cell isolation and separation technique provides highly specific single-cell separation, the output obtained is quite limited compared with that of FACS integrated with flow cytometry. However, specific single-cell separation techniques prevent tissue loss, allow searching in the tissue until the target cell is found, and are suitable for the separation of rare cells (Edgley et al., 2010; Minakshi et al., 2019).

2.6. Raman Spectroscopy

Although this method is not primarily used for cell separation, it is included in this section because it allows chemical analysis on the basis of certain chemical properties of the cell.

Raman spectroscopy is a noninvasive optical spectroscopy method based on the "Raman principle" established by the Indian scientist Chandrasekhara Venkata Raman. In 1928, he first reported that when substances were illuminated with monochromatic coherent light, they scattered light of different colors. He attributed this result to the frequency shift in the scattered light and defined it as a "new type of radiation" distinct from fluorescence. It was demonstrated that the vibrational movements of molecules in a sample were responsible for this phenomenon, and in 1950, "Raman spectroscopy" was developed, paving the way for its use in chemical analysis. According to this principle, in simple terms, a small portion of monochromatic light incident on an object is scattered from the sample with a frequency and thus color, which is different from that of the illuminating light. This difference arises from inelastic scattering due to the vibration of molecules in the illuminated substance. Since different substances have different Raman shift frequencies, detailed information about the chemical structure of the illuminated sample can be obtained (Raman, 1928; Ferraro et al., 2003; Akçe & Kadıoğlu, 2020).

Along with advancements in laser sources, progress in computer and imaging systems has led to the development of Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopes. These microscopes can reveal the quantity of certain compounds in cells through imaging without applying staining procedures. This allows for the rapid separation and diagnosis of normal and unstained neoplastic cells (McCullagh et al., 2022).

Although a version of the system specifically applicable to cell typing and cell separation has not yet been developed, its most exciting potential application is the *in ovo* sex determination of chicken embryos very early in incubation. The system exploits several biological phenomena: first, chicken erythrocytes are nucleated and represent the earliest blood cells formed during embryonic hematopoiesis; second, among chicken sex chromosomes, the Z chromosome is larger than the W chromosome. In poultry, the male genotype is homogametic (ZZ) and contains approximately three times more DNA than the female genotype (ZW).

2.7. Cell Adhesion

Some cells attach to molecules on the culture surface through cell membrane adhesion molecules (CAMs). On the basis of their adherence properties, cells can be categorized into three groups: adherent cells, suspension-forming cells, and cancer cells, which do not adhere to the surface but instead form amorphous aggregates or spheroid clusters in the medium. Owing to the ability of certain cells to attach to specific materials, selective cell cultures for particular cell types can be established. Coating the surface of a material with specific proteins can further increase the selectivity of the system.

In adherent culture methods, macrophages, fibroblasts, and mesenchymal cells can be separated. For example, after mononuclear cells are isolated from peripheral blood samples, they are plated in polystyrene culture dishes coated with serum and a cytokine mixture that promotes monocyte/macrophage differentiation. After 5–7 days of culture, the macrophages differentiated and formed a monolayer at the bottom of the culture dish. Microglia can be separated in a similar way.

In systems where adherent culture is applied in a negative separation manner, the targeted cells remain in suspension, while unwanted cells adhere to the surface. An example is the separation of respiratory tract epithelial cells, which remain nonadherent, from adherent fibroblasts.

Adhesion-based cell separation techniques are relatively simple, reproducible, cost-effective, and allow the collection of large numbers of cells. However, the purity of the obtained cell populations is generally low, and the risk of contamination with other cell types and bacteria is considerably high (Foster, 1996; Barlow & Huntley, 2000; Ergüler et al., 2002).

2.8. Cell Affinity Chromatography

The principle of affinity chromatography is based on the specific and reversible binding of the target biomolecule to ligands that have complementary binding sites and are immobilized on an insoluble support material (matrix). Affinity chromatography based on molecular recognition is essentially a technique that enables the purification of proteins (Wilchek & Chaiken, 2000; Zou et al., 2001). This technique is also applied in the separation of many molecules, such as proteins, carbohydrates, enzymes, and vitamins, and in the purification of antigens and antibodies (Hage & Cazes, 2005). Affinity chromatography has significant advantages over other techniques because of its high selectivity, speed, and ease of application.

The mechanism of specific cell separation by cell affinity chromatography is based on the interaction between cell surface receptors and molecules on a fixed surface. In this binding, the specific noncovalent attachment of macro molecules on the cell surface to the bonds in the stationary phase plays a role (Zou et al., 2001).

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