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PREPARATION OF REAGENTS AND CALIBRATION OF THE SINGLE RADIAL IMMUNODIFFUSION TEST (IDSR) FOR TITRATION OF TETANUS TOXOID

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Abstract

Antigen was prepared using mature culture of Clostridium tetani by formalin inactivation and heat, precipitation and ammonium sulfate concentration and dialysis against 0.5 M phosphate buffered saline was performed 4 hyperimmunization New Zealand breed rabbits weighing 2.5 Kg and obtained rabbit serum anti-Clostridium tetani. Rabbit serum anti-Clostridium tetani process and then received gamma globulin and tetanus immunoglobulin G (IgG) tetanus. Serum was obtained by Clostridium tetani sheep anti-IgG antibody which was used as the test IDSR. Were collected, processed and analyzed blood sera from immunized horses. 2% Noble agar was used in phosphate buffered saline (PBS) and serum anti-sheep IgG diluted C. tetani and 56^{o}C incorporated into the reaction microplate was made with a diameter of 5 cm. After solidification to a well practiced central and 5 peripheral wells. The central well was submitted serum sample with known titer standard and in peripheral samples analyzed. Reading and interpreting the reaction was performed at 24 hours. It was established standard curve necessary for interpreting the results. The results were expressed in international antitoxic units (UAI). We have tested a total of 30 blood sera samples collected from horses immunized IDSR assay and in vivo neutralization test white mice (SN), and comparing the results. In all cases determined by IDSR titers corresponded to those obtained by SN on white mice. The obtained results indicate that this assay can be used to determine the tetanus antitoxin titer at each harvest and intermediate product (in bulk) for the manufacture of anti-tetanus serum.

Keywords*: IDSR test, standard curve, tetanus antitoxin titer*

Introduction

Simple radial immuno assay (IDSR) or the method Mancini, Heremans, Carbonara is widely used in quantitative estimation of antigens. This test is more sensitive than Ouchterlony double immunodiffusion in gel by incorporating antiserum. The antigen is allowed to diffuse into agar wells practiced. The diameter of the zone of precipitation is directly proportional to the concentration of antiserum, and inversely proportional to the molecular weight, the concentration of antibody and the height of the gel on the plate [1]. Liunggvist et al. applied the IDSR test for the presence of

tetanus toxin in the vaccine series. This method is routine, and is used as an alternative to the test of flocculation [4]. This was used in comparison with the immunoelectrophoresis test "rocket" and determining the concentration of flocculation reaction of diphtheria toxin and toxoid. Differences between tests were not significant [4]. El Meshad chickens immunized with tetanus toxin IgY antibody titer obtained from the yolk was determined by IDSR. The method proved to be sensitive and specific for tetanus toxin titration [3]. IDSR used to quantify the staphylococcal toxin proved to be a specific and reproducible method, its sensitivity is 5 µg/ml [7]. IDSR test was used to quantitate the production control process of anti-rabies vaccines. The experiments were carried out to IgY anti-viral glycoprotein. Standard dose-response curve was performed according to WHO standards using standard rabies vaccine (NIBSC). The results were interpreted by the "Slope Ratio Standard Assay" (SSRA). Between values of standard vaccine and vaccine series there was a positive correlation coefficient of Pearson (r) is between 0.96 and 0.99 (2.56).

Material and methods

Samples investigated: tetanus serum, serum from horses immunized
Animals: New Zealand rabbits, sheep

Materials and equipment needed

Substances: Agar Noble or agarose, veronal sodium, sodium acetate, hydrochloric acid, sodium mertiolate.

Reagents: Clostridium tetani anti-IgG sheep serum, immunoplastics (5 cm diameter polystyrene petri dishes containing Clostridium tetani anti-IgG sheep serum agar gel), reference horse anti-tetanus serum (titre IAU known) Laboratory Objects: 5 cm diameter petri dishes, 3 mm diameter well mold, 15/160 mm tubes, wet chamber, 20 µl fixed or adjustable micropipettes, 200 µl and 1000 µl, graduated pipettes of 2ml and 5 ml.

Apparatus: Analytical balance, Marie bath, ultrathermostat set at 56^{o}C, negatoscope, centrifuge

Procedure

Veronal buffer pH 8.6 is prepared: Veronal sodium 9.78g, Sodium acetate 6.47g, Sodium mertiolate 0.1g, N/1 hydrochloric acid 6ml, 1000ml bidistilled water. The buffer thus prepared is diluted when used with 1: 1 bidistilled water and the pH is adjusted to 8.6.

Prepare the agar gel: add 2 g of agar to 100 ml of buffer. The mixture is boiled in a water bath until the agar has been completely dissolved, agitating the vial from time to time. Dispense 2 ml of hot agar and shake at 56°C in about 15 minutes.

Prepare the Clostridium tetani anti-IgG sheep serum: the Clostridium tetani anti-IgG sheep serum is diluted separately with a pH 8.6 veronal swab and kept at 56°C for 15 minutes. Dilution of the antiserum is chosen according to its titre and is indicated by the manufacturer. In our case we used as antigen 1.2 ml Clostridium tetani anti-IgG sheep serum and 0.8 ml buffer. Mix 2 ml of the 2% agar gel with 2 ml diluted Clostridium tetani anti-IgG serum. The agar-antiserum mixture is made by pouring the contents of the antiserum into the agar tube, both kept at 56°C, and gently agitating for homogenization

Prepare the reaction plates: the obtained mixture quickly pours into the plates placed on a perfectly horizontal surface and prevents the formation of air bubbles. The plates are left for about 30 minutes at room temperature for complete agglomeration of the agar. In the summer time put 15 minutes in the refrigerator. Plates so prepared can be stored for 3 months at 4^{o}C in plastic bags, hermetically sealed with lid to avoid wetting the agar layer with condensation water. In the agar gel, a central well and five peripheral wells, 3 mm in diameter, 1-1.5 cm from the edge of the plate are applied to the matrix. The agar gel is aspirated from each well.

Assignment of reagents: place 7 µl of the reference anti-serum serum in the central well. In the peripheral wells is distributed 7 µl of the samples to be examined. Filling of the wells and subsequent manipulation of the immunoplasm must be done with caution so that the samples distributed in the wells do not exceed the edges of the wells. Plates were placed in the wet chamber and kept at 25°C for 24 hours.

Reading the reaction: done 24 hours using the negatoscope. The diameters of precipitation zones are measured in mm with a graduated transparent ruler.

Calculation of results and mode of expression: the anti-tetanus antibody concentration values in the samples to be analyzed are read directly on a standard curve, based on the magnitude of the precipitation diameters measured in mm. The concentration of anti-tetanus antibodies is expressed in international anti-toxic units (UAI)/ml. If the diameter of the reference serum precipitation zone is consistent with the IgG concentration indicated by the manufacturer, the IgG concentrations of the test samples are read directly on the standard curve. If the normal reference serum does not achieve the value that matches the diameter and concentration due to inadequate immune preservation, decalibrated micropipettes or pipetting

errors, a correction is applied. For example, if the immunoplate reads for the reference serum a diameter of 11.5 mm instead of 10.8 mm then it decreases for each examined sample 0.7mm and then reads the correspondence on the standard curve.

Results and discussion

Antigen was prepared using mature culture of Clostridium tetani by formalin inactivation and heat, precipitation and ammonium sulfate concentration and dialysis against 0.5 M phosphate buffered saline was performed 4 hyperimmunization New Zealand breed rabbits weighing 2.5 Kg and obtained rabbit serum anti-Clostridium tetani. Rabbit serum anti-Clostridium tetani process and then received gamma globulin and rabbit tetanus immunoglobulin G (IgG) tetanus. Rabbit tetanus immunoglobulin G (IgG) tetanus was inoculated into the sheep and obtained Clostridium tetani sheep anti-IgG antibody which was used as antigen in the IDSR test.

Purification of anti-Clostridium tetani IgG was performed by DEAE-cellulose ion exchange column chromatography (Fig. 1)

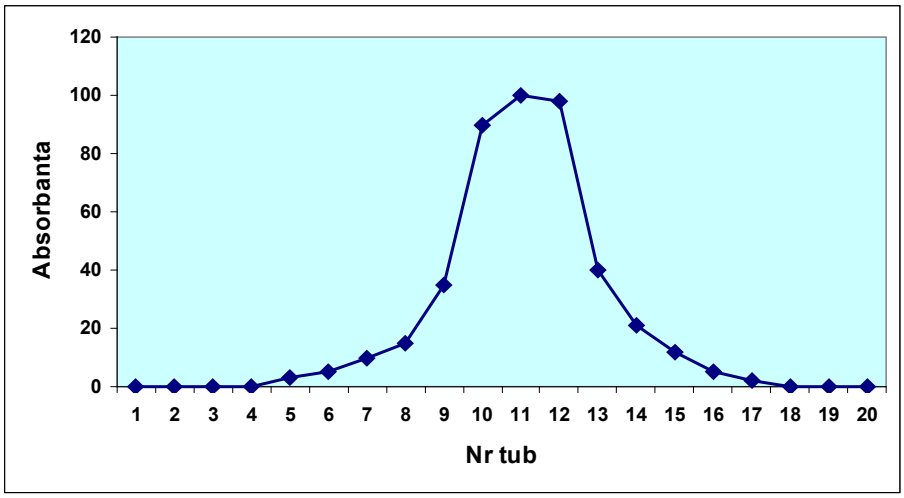

Fig.1. Elution profile of anti-C. tetani sheep IgG obtained on DEAE-cellulose

The peak containing fractions 8-14 were pooled and it was obtained anti-Clostridium tetani sheep IgG which was used as an antigen in the IDSR assay. Characterization of sheep IgG anti-Clostridium tetani was done by PAGE-SDS electrophoresis (Laemmli) using a SCIE-PLAS TV-100 apparatus, 10% polychrylamide gel, 0.1 M Tris-HCl separation buffer, pH

8.0, Tris-HCl concentration buffer 0 , 5M, pH 6.8, Tris-glycine migration buffer, pH 8.3 and Commassie Brilliant Blue, 0.1% (Fig. 2) and Immunoelectrophoresis (Fig. 3).

Were collected, processed and analyzed blood sera from immunized horses. 2% Noble agar was used in phosphate buffered saline (PBS) and serum anti-sheep IgG diluted C. tetani and 56^{o}C incorporated into the reaction microplate was made with a diameter of 5 cm. After solidification to a well practiced central and 5 peripheral wells. The central well was submitted serum sample with known titer standard and in peripheral samples analyzed. Reading and interpreting the reaction was performed at 24 hours (Fig. 4).

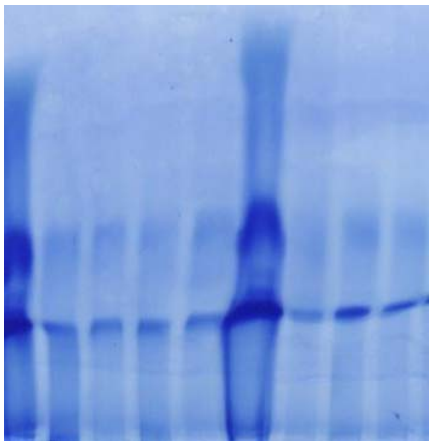

Fig. 2. Control PAGE-SDS IgG purified sheep

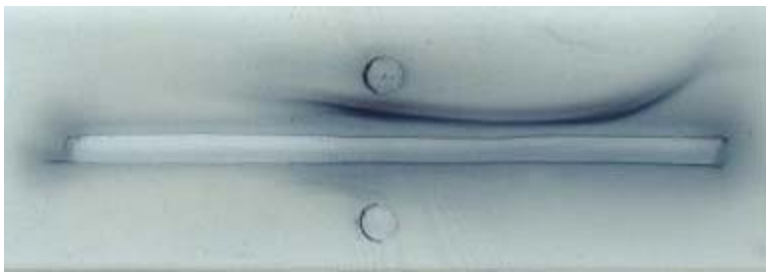

Fig. 3. Immunoglobulin G (IgG) anti C. tetani Purified Control Immunoelectrophoresis

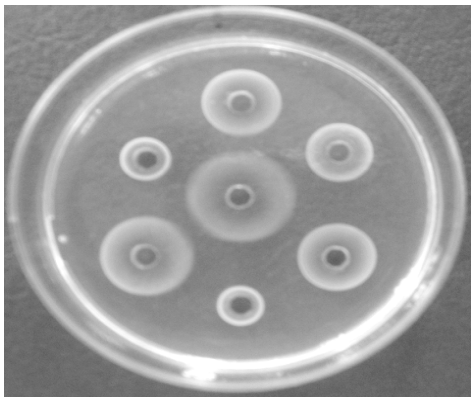

Fig. 4. Detection of tetanus antitoxin titre by the simple radial immunodiffusion test, immunoplates

It was established standard curve necessary for interpreting the results. The results were expressed in international antitoxic units (UAI) (Fig. 5).

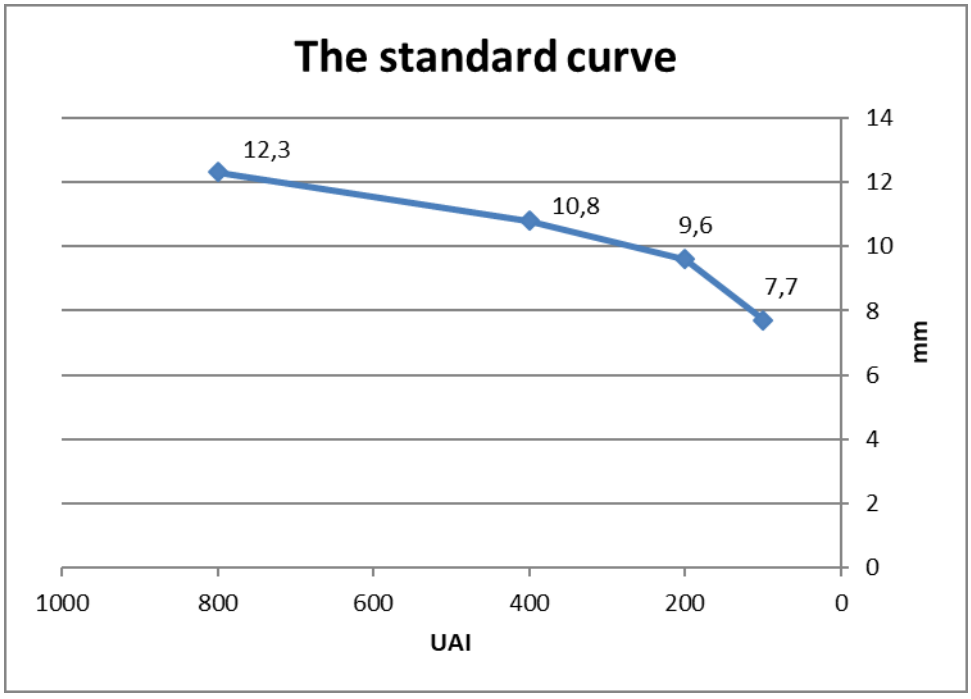

Fig. 5. The standard curve international anti-toxin units

We have tested a total of 30 blood sera samples collected from horses immunized IDSR assay and in vivo neutralization test white mice (SN), and comparing the results. In all cases determined by IDSR titers corresponded to those obtained by the serum neutralization test (SN) on white mice. The obtained results indicate that this assay can be used to determine the tetanus antitoxin titer at each harvest and intermediate product (in bulk) for the manufacture of anti-tetanus serum.

Conclusions

We have prepared and characterized the agents for the simple radial immunodiffusion test (IDSR) used to titrate the tetanus antitoxin.

For the determination of sensitivity, specificity and reproducibility, complementary tests were used: the serum neutralization test (SN) on white mice, Ramon flocculation reaction and double agar gel immunodiffusion test (ID).

The simple radial immunodiffusion test was found to be as sensitive, specific and reproducible as the seroneutralisation test performed on white mice and the Ramon flocculation reaction.

To perform the analysis, a very small sample quantity of 20 µl is required, and the reading and interpretation of the reaction takes place over 24 hours

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APPLICATIONS OF FOOD SAFETY FOR MEAT PRODUCTS

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Abstract

This paper presents various aspects related to the quality and safety of meat products. The paper is divided into two parts as follows:

In the first part of the paper, we presented the concept of food quality and safety. We defined the HACCP system and the terms used in its implementation. We also presented the principles and stages of HACCP, its scope, and its use in the meat product industry.

In the second part of the paper, the focus is on the application of the HACCP system in a meat product production unit. We discussed the varieties of products sold, described the technological process, the equipment used, and identified contamination risks. We also mentioned the physico-chemical analysis and sanitation tests.

Keywords: *food safety, meat products, control point, physico-chemical analysis, sanitation tests, technological process, HACCP.*

Introduction

This paper presents various aspects related to the quality and safety of food for meat products, the application of the HACCP system in the meat product industry, and general concepts about their production.

Food products, regardless of their origin, are necessary to provide the energy and nutrients required to support metabolism (energy, structural, catalytic roles, etc.), thereby maintaining tissue and organ functions, growth, reproduction, homeostasis, and the consumer's health status. Due to the influence of food products on the human body, it is important to ensure high-quality food to guarantee their safety for consumption. Due to potential risk factors that can affect the quality and safety of food, food inspection is necessary to ensure that products are wholesome and safe for consumption. The qualitative and hygienic value of food products can be compromised at any stage of the production processes due to various physical, chemical, or biological causes [2].

The nutritional value of food products is studied from an energetic perspective and through the content of macronutrients (caloric nutrients) and micronutrients (minerals, vitamins, enzymes, etc.). Nutritional compounds can be essential (cannot be synthesized by the body and must be obtained from the external environment through diet) or non-essential (can be

synthesized by the body through various metabolic processes). Examples of essential nutritional compounds include 9 amino acids (phenylalanine, tryptophan, leucine, lysine, etc.), 2 fatty acids (linoleic acid and alpha-linolenic acid), minerals, and vitamins. Examples of non-essential nutritional compounds include 15 amino acids (alanine, arginine, aspartic acid, taurine, tyrosine, etc.), as well as a large portion of carbohydrates and lipids [5].

Food safety is regulated, thereby establishing the necessary decisions to be implemented to ensure the quality and safety of food products. These decisions should be based on solid scientific knowledge and research that demonstrate their effectiveness."

Matherial and Method

This research aims to apply quality and food safety standards in a meat product manufacturing unit, along with verifying the products through laboratory methods and self-control (physicochemical and microbiological examinations).

The research was conducted in the second semester of 2023, during which examinations were carried out on 30 samples of meat products, including 15 samples of meat products in casings and 15 samples of specialty meat products.

These examinations consist of physicochemical and microbiological tests, as follows: Through physicochemical examinations, we assess the integrity of the meat products. These methods aim to determine the quantities of water, proteins, fats, salt, nitrogen compounds, and ammonia.

Bacteriological examinations are conducted to determine the presence and count of various potentially pathogenic microorganisms. Microbiological examinations are occasionally performed when contamination of the products is suspected. Sanitation tests are carried out periodically to control hygiene throughout the production process.

Sampling is done from critical control points (such as equipment, different surfaces, etc.). Sterile swabs provided in the kit are used, and after sampling, the swabs are placed in the tubes provided in the kit. The tubes are then incubated for a specified time at a temperature indicated in the kit's instructions [6].

Table 1. Results obtained from the physicochemical examination of meat products in casings in the second semester of 2023

No.	Product name	H2O %	NaCl %	Nitrite mg%	Easily hydrolyzable nitrogen mg/NH3/100g	Fat %	Protein %	Kreiss
1	Salam de vară	50,1	2,5	5,6	35,7	38,2	7,1	Neg.
2	Cabanos	51,6	1,8	5,1	38,4	28,4	17,7	Neg.
3	Cârnați de porc	51,0	1,7	4,2	36,3	21	15,7	Neg.
4	Salam Italian	61,5	2,4	4,4	36,4	28,7	16,9	Neg.
5	Salam Victoria	51,5	2,2	5,2	36,3	23,2	20,5	Neg.
6	Cârnați Grătărel	54,3	3,3	3,5	33,1	23,5	18,7	Neg.
7	Crenvurști	52,1	2,3	4,1	34,5	23,4	8,7	Neg.
8	Cârnați de porc	61,3	1,6	4,2	36,2	19	15,7	Neg.
9	Salam de vară	51,5	2,2	2,8	46,4	29,2	15,4	Neg.
10	Salam Italian	52,2	2,4	3,8	34,4	29,2	17,2	Neg.
11	Salam Victoria	53,2	2,5	4,3	31,8	25,2	16,7	Neg.
12	Cârnați Gratărel	52,5	2,3	8,4	32,8	23,4	18,2	Neg.
13	Cabanos	51,7	3,4	2,7	38,4	26,8	17,5	Neg.
14	Crenvurști	50,2	2,4	3,9	34,4	23,5	8,5	Neg.
15	Salam Victoria	51,3	5,8	5,3	36,2	23,2	20,5	Neg.

In addition to rapid sanitation tests, specific tests can be performed to detect certain bacteria such as E. coli, Listeria, Salmonella, fungi, yeasts, etc [1].

Results and discussion

Following the physicochemical examinations on a total of 15 samples of meat products in casings, variations in the normal physicochemical parameters were found in different varieties. These irregularities were found in the following products: Italian salami, summer salami, Victoria salami, Cabanos, pork sausages, Grătărel sausages (Table 1), and homemade pastrami (Table 2).

Table 2. The results obtained from the physicochemical examination of specialty meat products in the second semester of 2023

No.	Product name	NaCl %	Nitrite mg%	Easily hydrolyzable nitrogen mgNH3/100g	Kreiss reaction
1.	Jambon de porc	2.4	3.1	30.2	Negative
2.	Pastramă porc	4.1	4.2	34.2	Negative
3.	Pastramă casă	**5.6**	**7.5**	33.1	Negative
4.	Kaiser	2.2	3.3	23.1	Negative
5.	Mușchi file	2.9	3.4	22.1	Negative
6.	Mușchi țigănesc	2.8	3.7	21.3	Negative
7.	Șuncă de casă	2.7	3.0	21.5	Negative
8.	Ciolan afumat	2.6	3.0	33.1	Negative
9.	Costiță afumată	2.2	3.7	30.5	Negative
10.	Piept afumat	2.4	3.8	28.5	Negative
11.	Ceafă afumată	2.0	3.9	31.2	Negative
12.	Jambon de porc	2.5	3.0	30.3	Negative
13.	Pastramă porc	4.1	4.0	34.1	Negative
14.	Pastramă vită	4.3	4.2	34.8	Negative
15.	Kaiser	2.5	3.8	23.8	Negative

Table 3. Results of the microbiological examination of meat products (sausages)

Samples	Analyzed microorganisms						
	NTG	Coliform bacteria mo/g	Escherichia Coli, mo/g	Coagulase positive Staphylococcus mo/g	Salmonella mo/25g	Sulfite-reducing bacteria mo/g	Bacillus cereus, mo/g
1	0	61	2	1	Absent	41	Absent
2	0	63	1	1	Absent	40	Absent
3	0	52	3	3	Absent	42	Absent
4	0	53	2	2	Absent	50	Absent
5	0	51	4	1	Absent	43	Absent
6	0	44	1	2	Absent	50	Absent
7	0	45	2	1	Absent	42	Absent
8	0	52	3	3	Absent	50	Absent
9	0	48	1	2	Absent	51	Absent
10	0	45	2	4	Absent	45	Absent
Averag	-	51.4	1.9	1.9	0	45.4	-

Table 4. Results of the microbiological examination of work surfaces

Samples	Analyzed microorganisms						
	NTG	Coliform bacteria mo/g	Escherichia Coli, mo/g	Coagulase positive Staphylococcus mo/g	Salmonella mo/25g	Sulfite-reducing bacteria mo/g	Bacillus cereus, mo/g
1	1	-	0	0	-	0	0
2	1	-	0	0	-	0	0
3	1	-	0	0	-	0	0
4	1	-	0	0	-	0	0
5	1	-	0	0	-	0	0
6	1	-	0	0	-	0	0
7	2	-	0	0	-	0	0
8	2	-	0	0	-	0	0
9	1	-	0	0	-	0	0
10	1	-	0	0	-	0	0
Average	1.2	Absent	0	0	Absent	Absent	Absent

Conclusion and recommendations

Following the physicochemical examinations conducted in the last semester of 2023 on a total of 30 samples of meat products in casings and specialty products, certain deviations were found in specific finished products regarding the quantity of salt, nitrites, and slight variations in the percentage of water and easily hydrolyzable nitrogen [4].

The finished products in which deviations from normal physicochemical values were identified include Italian salami, summer salami, Victoria salami, Cabanos, pork sausages, Grătărel sausages (Table 1), and specialty homemade pastrami (Table 2).

The Kreiss reaction for freshness assessment was negative for all analyzed samples, indicating an adequate state of freshness of the finished products.

As a result of the microbiological examinations, based on the obtained results presented in Tables 3 and 4, it can be deduced that hygiene standards, good manufacturing practices, and efficient implementation of the HACCP system are followed throughout the production chain in the manufacturing unit.

The increase in physicochemical parameters related to the quantity of salt (NaCl) and nitrites indicates a desire by the producer to improve the organoleptic characteristics and shelf life of the finished products. However, due to exceeding the upper limit of these parameters, it is recommended to

adhere to the manufacturing recipes, accurately weigh auxiliary materials, and implement corrective actions from the HACCP plan [3].

In addition to following the recipes and technological procedures of manufacturing, periodic verification of equipment and working tools is recommended to ensure their proper functioning, thereby increasing productivity and meeting customer needs.

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THE IMPORTANCE OF INTRAOCULAR PRESSURE MEASUREMENT

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Abstract
The present case study emphasizes the importance of paraclinical examinations in the investigation of the visual apparatus in cats, especially the determination of IOP; often postponing or ignoring this investigation can cause irreversible damage to the eye, even the total loss of visual acuity.

Keywords: *anisocoria*, *intraocular pressure measurement, tonovet, uveitis*

Introduction

The case study wanted to highlight the importance of introducing intraocular pressure measurement into the daily routine of the clinical veterinarian, often, eye injuries can hide serious diseases, which cannot be diagnosed by clinical examination, therefore, the introduction of paraclinical examinations in ophthalmological examinations can help to restore vision and heal our feline patients.

Materials and methods

The case study involved an 8-month-old European cat, female, with no history of ocular, infectious or parasitic pathology that could, secondarily, influence the visual system. After performing the clinical examination by inspection and palpation, the paraclinical examination of the fundus and the measurement of intraocular pressure with the tonometer were performed - iCare TONOVET Plus – iCare Finland (Fig. 1).

Fig. 1. Examination kit

Results and discussion

An 8-month-old cat presented for consultation with blepharospasm. For the past 6 weeks, she had been treated locally with Tobrex drops, twice a day.

The clinical examination revealed miosis, the presence of keratosis precipitates in the aqueous humor and low IOP - 16mmHg.

The examination of the fundus could not be performed, completely after instillations with TROPICAMIDE and it was observed that the pupil does not dilate sufficiently, having an "unnatural" shape.

Fig. 2. OS - Intraocular pressure measuring

Figure 3 OS - Performing tonometry

When examining with a magnifying glass and flashlight, a change in the appearance of the iris was observed in the sense of its inflammation - iritis, which, extended to the ciliary body, can cause a decrease in UA production and a decrease of intraocular pressure (Fig. 1). Also, discoria (deformation of the pupil) was a red flag [1].

At the insistence of the owner who had an average budget and the possibility of performing a local treatment only twice a day, it was discussed about the use of a "very expensive" gel which he had been informed about in advance, at the beginning of the previous treatments.

However, the proposal was to perform an ocular ultrasound which revealed an obvious retinal detachment (Fig. 4 and Fig. 5) [4].

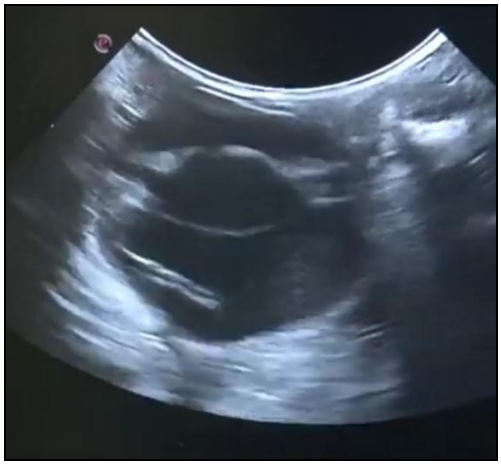

Fig. 4. OS - Ocular ultrasound

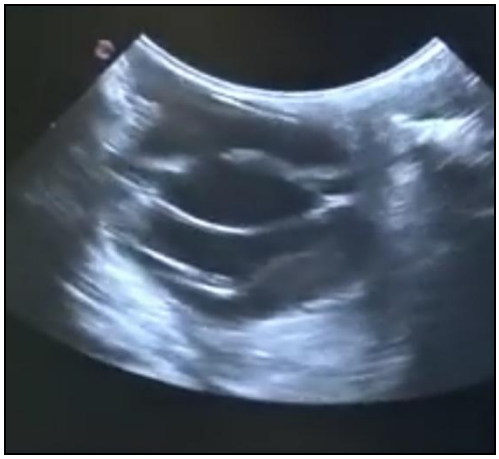

Fig. 5. OS - Retinal detachment

The diagnosis was completed by observing the following complementary indications:

- the cornea had a "blurred" appearance;
- short vessels, relatively straight, on the entire circumference of the sclero-corneal limbus;
- hypopyon in the anterior chamber (due to increased cellularity and fibrin – specific to inflammation);
- it's possible that the slightly unnatural appearance of the pupil and limited response to Tropicamide may have been caused by posterior synechiae (between iris and lens);
- it is very likely that the iritis and inflammation of the ciliary body continued at the level of the choroid and later at the level of the retina, the choroid being responsible for retina vascularization (Fig. 6);
- this can explain retinal detachment which is an irreversible condition due to which visual acuity is lost.

Unfortunately, for the present case, any treatment was late, the solutions being intrascleral prosthesis or enucleation of the eyeball, these being the only methods by which superinfections and pain can be removed.

It should be noted that, in early stages, uveitis (inflammation of the iris, ciliary body and choroid) can be treated with medication, locally and generally. Usually, locally, we choose Chloramphenicol, eye drops, Prednisolone 1%, and Atropine, and generally antibiotic (often Doxycycline) and anti-inflammatory for a few weeks.

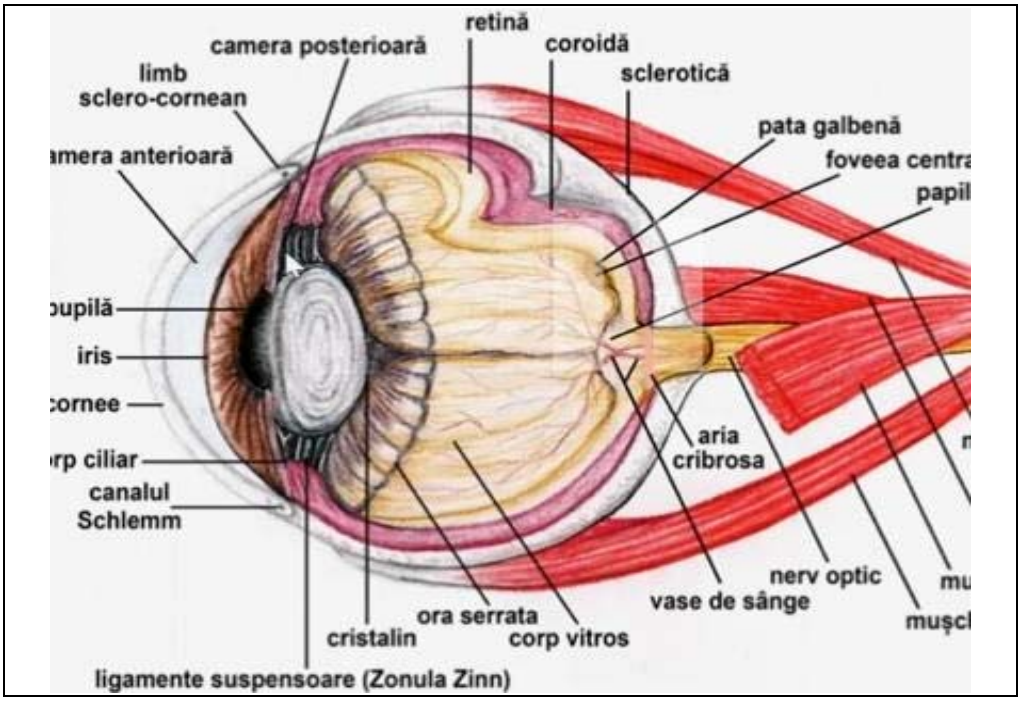

Fig. 6. Components of the eye (https://www.youtube.com/watch?v=-NZx0nTVVW0)

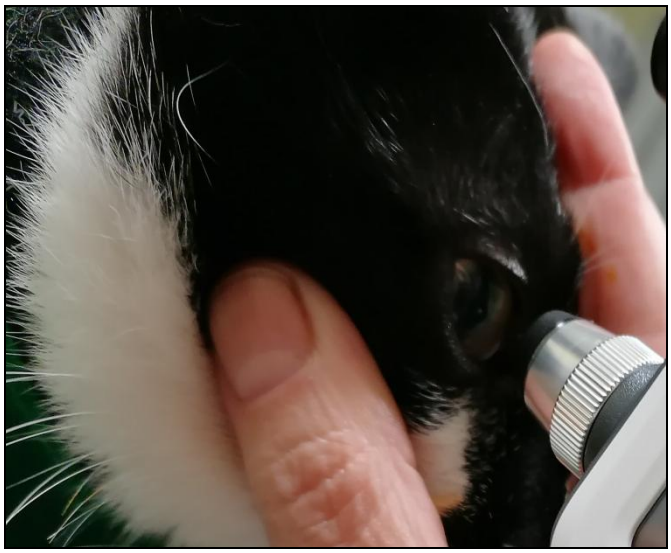

Fig. 7. OD - Intraocular pressure measuring

Although, in the present case, the IOP (Fig. 2) was a determining factor in establishing the uveitis diagnosis, a thorough clinical examination is very important, as it is possible that sometimes, due to fibrin deposits in the anterior chamber, the drainage of the aqueous humor may be prevented, and the intraocular pressure may have false values, being within physiological limits [6] [7].

The correct diagnosis is an excellent prognostic tool, which reduces the patient stress caused by an ineffective therapy (as in the present case), as well as unnecessary expenses of the owner.

Filling out the ophthalmology record must be done always after performing a minimum series of basic tests that consider:

- examination of the general appearance of both eyeballs, symmetry, size, bilateral pupil appearance;
- examination of the cornea using a magnifying glass, light and by performing the fluorescein test to detect possible corneal wounds;
- very important – the determination of IOP (Fig. 7) which can indicate a decrease in values in many cases, when we are dealing with penetrating wounds, as in the present case, probably extremely common in cats and with a clinical picture very similar to herpesvirus (for example);
- the ocular ultrasound is mandatory whenever the fundus cannot be examined.

In this case, the determination of IOP (Fig. 2; Fig. 3), which is part of the diagnostic routine tools, was the one that raised the first question mark regarding the fact that it may not be a simple "virosis" as the owners can often interpret in light of the fact that they have gone through similar situations with other patients [2] [3].

The use of multiple gels and collars "on demand" or "on budget", quickly worsens the clinical condition of the patient, and causes irreversible changes and total loss of vision.

Conclusions

1. Intraocular pressure measurement cannot be replaced by other clinical or paraclinical examinations.
2. Intraocular pressure provides essential information regarding both diagnosis and prognosis and treatment options.
3. Tonometry is a non-invasive, painless and quick method that should be included in the daily examination routine.

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THE PERIPHERAL BLOOD SMEAR - AN UNDERUSED AND UNDERVALUED DIAGNOSTIC TEST

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Abstract

The peripheral blood smear is frequently used in veterinary medicine as a diagnostic tool for diseases caused by rickettsial, protozoal and mycoplasmal agents. What practitioners often forget is that a blood film can also be used to examine the shape, size, number and color of erythrocytes, leukocytes, and platelets. This information is extremely valuable, and it can sometimes help guide the diagnosis and treatment without the need to run a multitude of expensive and time-consuming tests. This succinct review article does not include information related to parasitic or proliferative diseases. It simply aims to bring back the focus on a cheap and reliable diagnostic tool that can give a clinician additional data which might otherwise go unnoticed when relying solely on automated hematology analyzers.

Keywords: *peripheral blood smear, erythrocyte morphology, leukocyte morphology, platelets, blood, blood film*

Introduction

Automated hematology analyzers are widely used to produce fast and accurate complete blood counts. These devices offer a large variety of information regarding peripheral blood cells, including leukocyte counts (total number and differentiated counts), erythrocyte count, platelet count, hemoglobin concentration, packed cell volume and red blood indices. Some automated hematology analyzers can also provide information on platelet volume, red blood cell distribution width, and reticulocyte count.

However, it is important to acknowledge that these devices have a few limitations in veterinary medicine. Peripheral blood cell morphology varies from species to species, which may limit the use of some hematology analyzers for certain species [1, 2]. For example, the use of automated hematology analyzers is problematic for species with nucleated erythrocytes and thrombocytes. While there are certain automated cell counters and software dedicated for use in avian species, these devices and programs are not widely available, they require specialized training, and results continue to be unreliable [3, 4, 5, 6]. Another example is the high frequency of low platelet counts in cats, although thrombocytopenia is uncommon in this

species. A retrospective study [7] has analyzed 359 cases with low platelet counts in automated platelet counts and concluded that the apparent thrombocytopenia was attributed to platelet aggregation and the limitation of the medical device in discerning cell sizes.

The peripheral blood smear has an enormous diagnostic relevance in human medicine [8], but it might be even more relevant in the veterinary medical field. The complete blood count and the blood film are irreplaceable diagnostic tools that can aid a clinician in guiding a diagnosis, treatment course and treatment efficiency. Peripheral blood cell cytology is necessary as a quality control method when certain abnormal readings are noticed in an automated count such as a low platelet count in cats, high basophile counts in cats and dogs [9] and high lymphocyte counts in anemia patients. It is also vital in the differentiation of certain types of anemia, the diagnosis of myeloproliferative diseases, sepsis, hemoparasites, certain malignancies and genetic disorders. A blood smear should be ordered when a patient develops jaundice, is suspected of organ failure, nutritional deficiencies, intoxication or presents an enlarged spleen. The examination can be repeated at certain intervals for the evaluation of a patient's response to therapy.

Peripheral blood smear preparation

The quality of a blood smear will ensure the accuracy of interpretation. This is directly related to a few factors such as the experience of the medical staff realizing the blood smear, blood sample storage time and conditions, the anticoagulant used for blood collection, and the quality of staining dies.

A good quality blood film should be prepared within the first couple of hours of sample collection. Prolonged storage may cause artefactual changes such as platelet aggregation, loss of central pallor and crenation in red blood cells, cytoplasmatic vacuolation in neutrophils and monocytes and nuclear lobulation in lymphocytes. Storage artefacts can also translate to an increased MCV (mean cell volume) [10]. Using capillary blood will help avoid such artefacts.

The anticoagulant of choice for blood cell morphological analysis and the complete blood count using automated analyzers is EDTA (ethylenediaminetetraacetic acid) because it preserves cell morphology. There are certain exceptions in avian species like ostriches, black-crowned cranes and many *Corvidae* [11], as well as reptiles [12, 13, 14] where blood should be collected using heparin to prevent EDTA-induced hemolysis.

Blood films should uniformly cover about two thirds of the slide and should end with a round, feathered area. The whole slide should be analyzed

with low magnification for the detection of large bodies such as parasites (like *Dirofilaria)* or large blood clots. Cell morphology should be analyzed with high magnification towards the end of the film, where cells are organized in a single layer.

The standard stain used in hematology is a Romanowsky-type stain such as May-Grünwald Giemsa or Diff-Quick. Other stains are used for specific observation of reticulocytes, platelets, Heinz bodies or siderotic inclusions.

Artifacts can appear when using dirty slides, from the presence of fat droplets from fingerprints, or an improper slide preparation technique. Old staining dies can precipitate and an inexperienced examiner may have difficulties distinguishing this artefact from certain foreign bodies. Also, improper drying in a high humidity environment can cause crenation, refractile bodies that can be confused with parasites [15], and a sharper demarcation of the central pallor of red blood cells (punched out cells) which can impede the assessment of hypochromia [8]. These artefacts and the improper preparation of a peripheral blood smear can prove challenging when trying to interpret cell cytology. It should be noted that a new slide should be prepared instead of trying to examine a low-quality one.

Erythrocyte morphology

Red blood cell morphology can suffer size, shape, and color changes. Other important changes are the presence of cell inclusions and of young cells such as reticulocytes and metarubricytes. There are certain morphological aspects which can help identify and characterize the onset and progression of disease.

The presence of large numbers of reticulocytes, metarubricytes and Howell-Jolly bodies is indicative of a regenerative response to anemia. A basic complete blood count will only show limited information such as an increased mean cell volume and a decreased number of erythrocytes. Other aspects like an increase in the central pallor area of erythrocytes can be indicative of iron deficiency [16], pyridoxine and riboflavin deficiency [17], or prolonged copper deficiency [18], and will be translated by analyzers as a decreased mean cell hemoglobin concentration, mean cell volume and packed cell volume [16].

A blood smear examination can also help diagnose immune mediated hemolytic anemia, a type II hypersensitivity reaction. Morphological changes reported for this disease are related to the presence of spherocytes, ghost cells, and cell agglutination [19, 20, 21, 22]. Erythrocyte ghosts and spherocytes both correlated with hemolysis, but spherocytes are easily observed only in dogs, because of the clear disappearance of central pallor

in red blood cells. An osmotic fragility test can be performed for the species that don't have an observable central pallor [23]. A regenerative response may or may not be present [17, 24].

Red blood cell size changes can be observed in macrocytic anemias like the megaloblastic anemia caused by a deficiency in cobalamin and folic acid, with a higher frequency in cats and certain dog breeds like the Giant Schnauzer, Border Collie, Beagle, Chinese Shar-Pei, and Australian Shepard with a congenital B12 deficiency [25, 26, 27, 28].

Membrane defects can be observed in certain congenital diseases such as hereditary spherocytosis observed in cattle [29] and stomatocytosis observed in some dog breeds like Beagles, Australian Cattle dogs, Alaskan Malamutes, Drentse Patrijshounds, and Schnauzers [30, 31].

A very dramatic morphological find is the presence of schistocytes or fragmented erythrocytes, characteristic of microangiopathic hemolytic anemias. In this case, red blood cells are physically damaged and can appear as literal fragments with various shapes. The physical injury can occur when erythrocytes pass through fibrin clots, tumors, or damaged blood vessels. This is a common find in diseases like disseminated intravascular coagulation, Dirofilariasis, various tumors, spleen diseases, hemolytic-uremic syndrome or intravenous catheters [32, 33, 34].

Other red blood cell morphological changes are a direct result of the toxic effect of medication, venom or other substances. Oxidative damage can cause irreversible damage to erythrocyte membrane and cytoplasm leading to the formation of Heinz bodies and eccentrocytes. Some examples of oxidating factors are *Allium spp* poisonings in cats, dogs, sheep, cattle, horses and water buffalo [35, 36], acetaminophen in cats and dogs [37, 38], zinc poisoning in dogs [39], skunk musk spray in dogs [40], vitamin K antagonists, ketoacidotic diabetes, T-cell lymphoma [41], benzocaine, vitamin K, phenylhydrazine [42], methylene blue [43], and propofol [44].

Lead toxicity can also be observed in peripheral blood cytology. Lead poisoning causes anemia with significant rubricytosis and basophilic stipling. It has been characterized many species, including humans, small rodents [45], dogs [46], cats [47], and rabbits [48].

Lastly, there are some shape changes such as target cells, acanthocytes that are related to alterations in cell membrane cholesterol and have been linked to liver disease and tumors like hemangiosarcoma, osteosarcoma and lymphoma [49, 50].

Leukocyte morphology

Leukocytes can suffer many morphological changes related to size, appearance, maturation level and various inclusions. These changes can be useful in assessing a patient's health status, and one of the most useful finds is the left shift. This term refers to the presence of a large number of immature neutrophils, usually band neutrophils but also metamyelocytes and sometimes even earlier progenitors. The opposite of this is the right shift which describes the presence of hypersegmented neutrophils, and it's most associated with high production or administration of glucocorticoids, or prolonged storage of blood samples [51].

The neutrophil can also suffer from various toxic changes during an inflammatory response. Despite the name, these morphological changes are caused by an altered cell production during a time of high demand, and are represented by cytoplasmatic basophilia, cytoplasmatic vacuole formation, and the formation of Döhle bodies [52]. Toxic changes can sometimes be the only indication of an inflammatory response in the absence of neutrophilia or left shift, but they can also be a consequence of prolonged blood sample storage [53, 54].

Lymphocytes morphology can also be altered, with observable changes such as cytoplasmatic vacuolation in certain poisonings and metabolic diseases [55, 56], and reactive lymphocytes which are antigenically stimulated and can have a very dark-blue cytoplasm, various sizes and a small number of vacuoles [57].

Some morphological changes are indicative of specific anomalies and disorders. Pelger-Huë anomaly is characterized by a disorderly maturation of myeloid cells, in particular granulocytes and monocytes. It was diagnosed in many species including cats, dogs, and horses, and can be recognized by the presence of hyposegmented cells with coarse mature chromatin [58, 59, 60]. Another example is Chédiak-Higashi syndrome, described in humans, cattle, cats, mice, foxes, and mink [61, 62]. It is an autosomal recessive disorder with multi-organ implications characterized by abnormal platelet function, large pink granules in neutrophil and lymphocyte cytoplasm, and enlarged eosinophilic granules [63]. Lastly, lysosomal storage disorders like mucopolysaccharidosis can also cause changes in leukocyte morphology. It is characterized by the presence of vacuoles and dark to pink-violet colored granules called Alder-Reilly bodies in the cytoplasm of neutrophils and lymphocytes [64, 65].

Platelets

A blood smear should be used to ensure the accuracy of automated hematology analyzers if a complete blood count reveals thrombocytopenia. Artefactual thrombocytopenia is a frequent find in veterinary medicine and is related to platelet activation and aggregation during blood sample collection. In this case, blood cell cytology will reveal multiple clumps of platelets positioned predominantly towards the feathered edge of the smear.

A particular case of thrombocytopenia is observed in certain breeds of dogs like the Cavalier King Charles Spaniel [66, 67], Akita [68], Norfolk Terriers, and Cairn Terriers [69]. This inherited platelet disorder is asymptomatic, and it is characterized by macrothrombocytopenia, in which patients have a low platelet count, but with an increased platelet volume. In some cases, platelets have also been described as appearing elongated [68].

Thrombocytopenia can also be related to various types of inflammation, raging from immune-mediated diseases [69] to endotoxiemia [70] and parasitic infections [71]. Changes in platelet size and appearance can give the examiner information about the bone marrow's response. Some types of automated blood counters provide platelet indices like the *mean platelet volume* and *platelet distribution width* but are not widely available for clinicians with small practices. These morphological changes can be observed in a routine blood smear, and it is important to know that an increase in platelet volume can be a sign of platelet activation and an increased production of platelets in the bone marrow [72, 73, 74, 75].

Conclusion

Peripheral blood smear examination is a cost-efficient test that can provide a vast array of information, with a minimal use of resources that are readily available in most small practices. The change in peripheral blood cell morphology can help guide a clinician in making a diagnosis or establishing the efficiency of a treatment course.

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