The Influence of Temperature and pH on the Results Obtained in the Diagnosis of Avian Influenza and Newcastle Disease

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Abstract
Avian Influenza is an infectious, contagious disease of an acute evolutionary type with an epizootic enzootic character, affecting numerous species of domestic and wild birds, clinically characterized by serious general disorders, accompanied by respiratory, digestive, nervous phenomena and an edema of the subcutaneous connective tissue from head and neck region, and morphopathologically through hemorrhagic lesions in various tissues and organs. At present it is spread in most European countries, America, Asia and Africa. It is of great economic importance due to its very high morbidity and mortality. Because the disease is underdiagnosed, and people do not notice the illness or mortality occurring in their livestock, the virus is very difficult to control. The number of cases of infection increases in the spring and autumn due to the migrations that migratory birds perform. They either start the migration already infected, or become infected during the migration, in the stopovers where the disease develops. Infected birds do not manage to reach the end of the migration, the body being weakened due to the fight against the virus, they can no longer fly and end up dying in the territories of the free countries, leading to new outbreaks of Avian Influenza.

Keywords: Avian Influenza, diagnosis, Newcastle disease, pH, temperature.

1. Introduction

Many diseases with animal-to-human transmissibility have been known since ancient times, but the Pasteurian period, which was by far the turning point in the development of microbiology, is the peak of the accumulation, substantiation and detection of the causality and pathogenesis of many of them [1, 2].
Humans can become ill through contact with a multitude of pathogens of a viral, bacterial, parasitic and, last but not least, prion nature, which can be transmitted from animals to humans and vice versa [2-5].
Avian Influenza is an infectious, contagious disease of acute evolutionary type with epizootic enzootic character, affecting numerous species of domestic and wild birds, characterised clinically by severe general disorders accompanied by respiratory, digestive, nervous phenomena and oedema of the subcutaneous connective tissue in the head and neck region, and morpho-pathologically through haemorrhagic lesions in various tissues and organs [6, 7].
Avian influenza has been reported, with variable incidence, on all continents. It is currently prevalent in most European countries, America, Asia and Africa. It is of great economic

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importance because of its very high morbidity and mortality. As a zoonosis, relatively few cases of infection have been reported in humans [8-10].
Because the disease is under-diagnosed and people do not report illness or mortality in their household livestock, the virus is very difficult to control [11, 12].
The number of cases increases in spring and autumn because of the migratory birds' movements. They either start their migration already infected or become infected during migration at stopover sites where the disease occurs. Infected birds do not make it to the end of their migration, as their bodies are weakened from fighting the virus, they can no longer fly and end up dying in the territories of disease-free countries, leading to new outbreaks of Avian Influenza [13, 14].
In this paper, the results of the diagnostic work on Avian Influenza cases in birds are presented, with details of the working method used in the laboratory. The laboratory diagnosis was carried out in a Sanitary Veterinary and Food Safety Laboratory and the results are presented for each type of parameter [15].

2. Materials and methods

The study was carried out in a Veterinary and Food Safety Laboratory (VFSL), virology department and aimed at two objectives:
a) Effects of pH and temperature variation in the haemagglutination inhibition reaction used in the diagnosis of Avian Influenza, step 1: Determination of haemagglutinating antigen titer;
(b) Effects of pH and temperature variation on the haemagglutination inhibition reaction used in the diagnosis of avian influenza, step 2: Determination of haemagglutinating titre of sera.
For both parameters, international reagents provided by the International Reference Laboratory for Avian Influenza and Newcastle Disease were used and buffer solutions were prepared in the virology laboratory.
The antigen used in the detection of avian influenza virus antibodies by the haemagglutination inhibition reaction is prepared from the allantoic fluid of germ-free embryonated chicken eggs infected with the A/ck/Scotland/1/59(H5N1) virus strain.
Five combinations of avian influenza virus antigens are used for the diagnosis of avian influenza by the haemagglutination inhibition method in the country's Veterinary and Food Safety Directorates:
- Haemagglutinin 5 combined with Neuraminidase 1;
- Hemagglutinin 5 combined with Neuraminidase 3;
- Hemagglutinin 5 combined with Neuraminidase 8;
- Hemagglutinin 7 combined with Neuraminidase 1;
- Hemagglutinin 7 combined with Neuraminidase 7.
In the present work, antigen with Hemagglutinin 5 and Neuraminidase 1 will be used.
Positive serum used in the detection of avian influenza virus antibodies by inhibition of the haemagglutination reaction is prepared from blood serum collected from germ-free chickens following inoculation with the inactivated virus strain A/ck/Scotland/1/59(H5N1).
Five combinations of avian influenza virus antibodies are used in the country's Veterinary and Food Safety Directorates for the diagnosis of avian influenza using the haemagglutination inhibition method to validate antigens from the same combination:
- Positive serum for Haemagglutinin 5 combined with Neuraminidase 1;
- Positive serum for Hemagglutinin 5 combined with Neuraminidase 3;
- Positive serum for Hemagglutinin 5 combined with Neuraminidase 8;
- Positive serum for Hemagglutinin 7 combined with Neuraminidase 1;
- Positive serum for Hemagglutinin 7 combined with Neuraminidase 7.
In the present study, Positive Serum with antibodies for Hemagglutinin 5 and Neuraminidase 1 was used.
Negative Serum used in the detection of Avian Influenza virus antibodies by haemagglutination inhibition reaction is prepared from blood serum collected from germ-free chickens.
The used methods were based on general procedures, specific procedures and work instructions, general laboratory procedures and specific virusology laboratory procedures, i.e. "Detection of avian influenza virus antibodies via haemagglutination inhibition reaction".

Detection of Anti-Avian Influenza virus antibodies using the haemagglutination inhibition reaction

The method is based on the property of Avian Influenza viruses to exhibit haemagglutinating activity towards avian red blood cells. Inhibition

of haemagglutination indicates the presence of avian influenza A-specific antibodies. In the diagnosis of avian influenza, the RIHA test is used as a serological method for the detection of antibodies to influenza A virus of the family Orthomyxoviridae in the blood serum of birds as an indicator of infection with influenza A virus: A, B and C. Only Influenza virus type A infects birds [15, 8, 16].

Materials:

Reagents:

• H5N1 antigen (Haemagglutinin 5 combined with Neuraminidase 1) supplied by the International Reference Laboratory for Avian Influenza and Newcastle Disease, batch 11/18, shelf life 12/2023

• H5N1 Positive Serum (Haemagglutinin 5 combined with Neuraminidase 1) supplied by the International Reference Laboratory for Avian Influenza and Newcastle Disease, batch 2/18, shelf life 01/2024

• Negative Serum, germ-free, supplied by the International Reference Laboratory for Avian Influenza and Newcastle Disease, lot 1/17, shelf life 01/2026

Buffer solutions:

• phosphate buffer saline (TFS) pH 7.2±0.2
• 1% and 10% red cell suspension;
• sanitary alcohol;
• sterile distilled water.
• Alsever solution;
• Red blood cell suspension - rooster red blood cell suspension - collection in Alsever's solution at a ratio of 1:1 from a minimum of three SPF birds (if this is not possible, collect from regularly monitored SPF birds that are free of avian influenza antibodies). Blood is centrifuged in phosphate buffer saline pH = 7.2±0.2 for 10 minutes at 1700-2000 rpm three times. If the blood is from birds with avian pseudo-plague antibodies, wash 8-10 times.

Prepare a suspension of 1% red cells in phosphate buffered saline pH=7.2±0.2. Erythrocytes can be stored at 4°C for 4-5 days in Alsever solution. In the case of serum from species other than gallinaceous, a further 10% red cell suspension is prepared.

Necessary devices are presented in Table 1.

Other materials:

- V-bottom microtiter plates;
- tips for 10-50µl, 20-200µl and 500-1000µl semi-automatic pipettes;
- sterile reagent cuvettes;
- 15 ml centrifuge tubes;
- Laboratory glassware: graduated pipettes, Berzelius beakers, graduated cylinders, Erlenmeyer flasks, graduated flasks with stoppers;
- cotton wool;
- syringes;
- sanitary alcohol, rubber gloves, decontamination solutions (Vircon's).

Examined material:

In accordance with the World Organisation for Animal Health Standard 17025 and the Guidelines for the Diagnosis of Avian Influenza and Newcastle Disease, the entire reaction is carried out at a temperature of 20ºC±2ºC and a pH of 7.2±0.2.

Table 1. Devices required to perform RIHA

Device name	Device code
Ph-meter WTW	05420206
Hettich centrifuge	005585-02-00
Freezer	10004
Single-channel pipette	H34008252
Single-channel pipette	03A6002
Single-channel pipette	910484
Multichannel pipette	BU45065
Multichannel pipette	09Z8720
Multichannel Pipette	BU45065
Multichannel Pipette	KL702291
Vortex Stirrer	090729874/3008905

In the present paper we will use:

• H5N1 antigen, lot 11/18, shelf life 12/2023, in multiples of 7, monitoring 2 important parameters in the reaction, pH and temperature, for a higher standard deviation to identify risks occurred in the reaction, risks that may influence the quality of the final result;

• Depending on the result obtained in the antigen titration, the experiment is continued with the titration of Positive Serum, lot 2/18, shelf life 01/2024 and Negative Serum, lot 1/17, shelf life 01/2026, using under the same conditions, the parameters monitored in the antigen.

The blood is collected in 3 vials of 1:1 Alsever solution from a minimum of three birds free of Avian Influenza antibodies and the blood in each vial is washed with Phosphate Buffer Saline (PBS) with 3 pH values: 6.8, 7.2 and 7.6.

To adjust the pH and bring it to the desired values in this experiment, Sodium hydroxide, 1-Normal was used to raise it and hydrochloric acid 1-Normal was used to lower it.

It is very important that during washing, the vials are turned gently so as not to lysate the haem, resulting in haemolysis in the vial. If the red blood cells rupture, the procedure should be repeated from the first point, collection. Only rooster haem should be used. The difference in the amount of haem/ml between rooster and hen is 800000haem/ml. In the case of positive results with a very low titre (1/16), the probability of reporting an erroneous result using hen blood is very high.

After collection, the vials are centrifuged at 1900 rpm, after which the supernatant is extracted, made up with TFS (each vial with the corresponding TFS) and centrifuged again. This step is repeated 3 times. By doing this, any "trace" of antibody is removed from the outer wall of the red blood cells.

In the case of the first centrifugation, the supernatant (blood plasma) is in the upper part of the vial and the deposit of red cells with possible antibodies is in the lower part of the vial, then by successive washes the plasma and possible antibodies are removed, the supernatant is only TFS and in the deposit there are red cells free of antibodies.

After the blood wash has been completed, the preparation of the 1% red cell solution follows. Use 100 ml TFS with 0.05 grams of bovine albumin. Bovine albumin helps to bind the antibodies in the serum to the rooster red blood cells. Three solutions are made, each with a different pH.

The solutions can be kept refrigerated for up to 7 days.

Haemagglutination inhibition reaction to determine working titer:

- 0.025 ml TFS with albumin is dispensed into the first 4 rows of the 'V' bottom microtiter plate (A,B,C,D 1 - 12);

- 0.025 ml of virus suspension (infected allanto-amniotic fluid) is distributed in the first well of the first 4 rows;

- serial dilutions of 0.025 ml volume of virus suspension are run from the first to the last well;

- dispense 0.025 ml TFS into all wells (rows 1-4);

- dispense 0.025 ml of 1% red cell suspension into each well;

- shake the plate gently and leave it covered for 30 minutes at 20°C. During this time the red cells in the wells for the erythrocyte control, row D, should deposit at the bottom of the wells as a distinct button. The antigen titration is done on two rows.

In the experiment, antigen will be titrated in multiples of 7, (rows A-G), at 3 pH variations and 3 temperature variations, in V-bottom plates as shown below:

- Plate 1: -incubation temperature16ºC, pH 6,8
- Plate 2: -incubation temperature24ºC, pH 6,8
- Plate 3: -incubation temperature32ºC, pH 6,8
- Plate 4: -incubation temperature16ºC, pH 7,2
- Plate 5: -incubation temperature24ºC, pH 7,2
- Plate 6: -incubation temperature32ºC, pH 7,2
- Plate 7: -incubation temperature16ºC, pH 7,6
- Plate 8: -incubation temperature24ºC, pH 7,6
- Plate 9: -incubation temperature32ºC, pH 7,6

The plates are incubated in the thermostat, for 30 minutes, according to the temperature written on the edge of the plate.

In the case of antigen titration, buttons appear when antigen potency decreases considerably, through serial dilutions, leaving the hematite to migrate to the bottom of the well. When antigen potency is high, haematite is present in suspension.

The haemagglutination reaction is seen by tilting the plate with the observation of erythrocytes flowing down the well wall:

The haemagglutinating titre of the antigen is the highest dilution of virus that produces complete haemagglutination (without erythrocyte shedding). Reading is done by observing the presence or absence of haemagglutination:

- presence = fine deposits of red blood cell granules that will evenly coat the bottom of the well and the side walls,
- absence = haematites are deposited at the bottom of the well in the form of a well-formed button.

In the haemagglutination inhibition reaction, 4 UHA are used (e.g.: titer 256, resulting in a working solution of 1/64 i.e. 256/4 =64).
After 30 minutes of incubation, the following results are obtained:
according to the Specific Procedure and Guidelines for the Diagnosis of Avian Influenza and Newcastle Disease, to validate the antigen titre, the antigen must have either the titer indicated by the manufacturer, or with an over or under dilution. In this case, the manufacturer indicates a titre of 1/128.

3. Results and discussion

In the results obtained we can observe variations in titre under the same incubation and pH conditions, which indicates that the reaction is only stable in some situations (Table 2 and 3).

Table 2. Influence of temperature in the haemagglutination inhibition reaction to determine the working titer

Temperature influence on the reaction	Accepted (%)
Percentage of rows accepted at a temperature of 16 °C	58.78
Percentage of rows accepted at a temperature of 24 °C	25.45
Percentage of rows accepted at a temperature of 32 °C	15.77

Table 3. Influence of pH on the haemagglutination inhibition reaction to determine the working

Influence of pH in the reaction	Accepted	Measuring unit (%)
Percentage of rows accepted at a pH of 6.8	14.74	72
Percentage of rows accepted at a pH of 7.2	45.46	100
Percentage of rows accepted at a pH of 7.6	39.8	100

It is observed that the antigen is more stable at 16°C, falling within the standard limits of titer determination. Considering the results obtained for the temperature, it is concluded that incubation at a lower temperature than the temperature indicated on the leaflet gives the antigen a much better stability.
It is observed that the antigen is more stable at pH 7.2, falling within the standard limits of titer determination. Considering the results obtained for pH, it is concluded that a pH with a neutral or even basic value gives the antigen much greater stability.
Depending on the final antigen titer, we determine how much crude antigen to use in the dilution.
Ex. Titer: 256 divided by 4 i.e. 256/4 = 64
A plate has 96 wells. For each well 25 µl antigen solution is needed i.e. 25 µl X 96 = 2400~ 2500µl antigen solution.
If we have 10 plates to work with, this gives 10X 2500 µl antigen = 25000µl = 25 ml antigen according to the working titer.
25 ml : 64 = 0.39 ml antigen or 25,000µl : 64 = 390.62 µl crude antigen.
Of the 25000 µl TFS (required for the 10 plates) 390 µl TFS is aspirated and discarded. Add 390 µl crude antigen over the remaining TFS, this is the working antigen solution.
Thus, the following quantities are determined, depending on the dominant titre and pH (Table 4).

Table 4. Calculation of antigen for the reaction

Dominant titre	Amount/plate (ml)	pH	Plate identification no.	Crude antigen (ml)	Total amount (ml)
1/256	2.5	7.2	4	0.039	2.5
1/512	2.5	7.2	5, 6	0.039	5
1/512	2.5	7.6	7, 8, 9	0.059	7.5
1/512	2.5	6.8	1, 3	0.039	5
1/1024	2.5	7.6	2	0.01	2.5

At the end of the reaction the following serum titers are obtained:
Note that in order to verify the correctness of the titer established in the first step, it is verified by checking the haemagglutinating units in row H.

Thus, in the first well in row H, no haemagglutination occurs, in the second well there is a button but the flow is half and in the remaining wells there are tear shaped buttons. If in the second well we have a fully flowing button, the final titer will be one dilution higher and if the button is only half flowing in well number 3, the final titer will be one dilution lower.

Result interpretation is presented in Tables 5-8.

Table 5. Positive serum result interpretation

Percentage of acceptance of Positive Serum titers according to PSV 09	
Accepted	Not accepted
56%	44%

Table 6. Negative serum result interpretation

Percentage of acceptance of Negative Serum titers according to PSV 09	
Accepted	Not accepted
89%	11%

Table 7. Results reported to temperature

Temperature (°C)	Validated plates (%)	Not validated plates (%)	Identification of validated plates
16	66.66	33.33	1, 4
24	66.66	33.33	2, 5
32	33.33	66.66	3

Table 8. Results reported to pH

pH	Validated plates (%)	Not validated plates (%)	Identification of validated plates
6.8	100	0	1, 2, 3
7.2	66.66	33.33	4, 5
7.6	0	100	

At the end of 30 minutes and after results interpretation reported at the maximum allowed dilution, according to the Specific Procedure in the virology section and the Guidelines for the diagnosis of Avian Influenza and Newcastle Disease, 56% of the examined plates are valid for Positive Serum.

Following the expiration of 30 minutes and interpretation of the results reported at the maximum permitted dilution, according to the Specific Procedure in the virology section and the Guidelines for the Diagnosis of Avian Influenza and Newcastle Disease, 89% of the plates examined are valid for Negative Serum.

4. Conclusions

Diagnosis of avian influenza by the haemagglutination inhibition reaction in relation to the Specific Procedure and Guidelines for the Diagnosis of Avian Influenza and Newcastle Disease does not confer diagnostic stability in terms of temperature. The virology departments of the county veterinary and food safety directorates are not equipped with cooling devices in the wards, thus during the summer months the temperature in the ward exceeds 32°C. The experiment shows that a temperature higher than 24°C destabilises the antigen-antibody binding. At the same time, the pH of the phosphate buffer saline, gives the reaction stability at a neutral, slightly acidic value (6.8-7.2). In the case of reactions where the pH value of phosphate buffer saline is basic, the cell wall of the red blood cells ruptures and the antigen-antibody complex is not formed resulting in false positive reactions.

The conclusions that emerge are as follows:

The purpose of this paper has been achieved. Both temperature and pH influence and destabilize the reaction, leading to false negative or false positive reactions.

Validation of the plates at a different temperature and pH from the values found in the Specific Procedure and the Guidelines for the Diagnosis of Avian Influenza and Newcastle Disease gives much better stability and the margin of error in diagnosis is almost zero. Although the maximum dilution allowed for antigen titration (one dilution above or below the titer indicated by the manufacturer) does not allow passing to step 2

(serum titration) if this is exceeded, two dilutions above the indicated titer have been considered in this study, proving that in step 2 the serum titer is within the maximum dilution allowed by the Specific Procedure and Guidelines for the diagnosis of Avian Influenza and Newcastle Disease.

Thus, by accepting two extra dilutions, the amount of antigen used to prepare the working antigen solution is lower and therefore the costs are lower, with the same result.

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