

Development of Standard Reagents for Avian Influenza Virus Subtypes Diagnosis

Maria Angela Orsi¹, Soraya Cecilia Albieri Camillo¹, Eluana Carolina Fortunato¹, Christian Steffe Domingues¹, Dilmara Reischak¹, Margarida Maria Hoppner Zaroni¹, Tânia Rosária Pereira Freitas², Terra A. Jenson³ and Janice C. Pedersen³

1. Agriculture and Livestock National Laboratory-São Paulo, LANAGRO-SP, Ministry of Agriculture, Livestock and Supplay, MAPA, Campinas, São Paulo, Brazil

2. Agriculture and Livestock National Laboratory-Minas Gerais, LANAGRO-MG, Ministry of Agriculture, Livestock and Supplay, MAPA, Pedro Leopoldo, Minas Gerais, Brazil

3. U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Veterinary Services Laboratories (NVSL), Ames, IA, USA

Abstract: Avian influenza (AI) is one of the most relevant viruses in the poultry industry. The AI virus (AIV) transmission from birds to human causing severe cases and mortality enhanced the magnitude of AI for public health. Consequently, the AIV diagnosis laboratories should be able to detect and identify endemic, epidemic and seasonal influenza strains and other wildlife influenza subtypes that cross the country's borders. The development in quality controls in according with international rules comes to improve the performance of tests. With this purpose, the Brazilian Reference Laboratory for Avian Influenza (LANAGRO-SP) established a cooperation with the World Organization for Animal Health (OIE) to produce AIV master seeds, inactivated antigens and antiserum to attend the necessities of Brazil and other South America countries under the high quality control for all test. Seventeen of AIV master seed lots and seventeen of inactivated antigens lots produced reached hemagglutination (HA) titers of 1:512 and 1:256, respectively. In addition, fifteen AIV antiserum lots with hemagglutination inhibition (HI) titers reaching 1:4,096 were obtained. The AIV reference reagents produced and applied in laboratory routine successfully.

Key words: Avian influenza virus, reference reagents, subtypes, quality control, Brazil.

1. Introduction

The Brazilian poultry industry has conquered a relevant position as one of the largest chicken meat exporters and the second place of the poultry producers in the world [1]. Therefore, the Brazilian Ministry of Agriculture, Livestock and Food Supplies (MAPA) investments in the effective monitoring and control programs for avian high-risk diseases and diseases subject to notification as avian influenza (AI) are relevant either to Brazilian as to the other countries in South America avian producers [2].

AI, a bird infirmity caused by the AI virus (AIV)

type-A occurring naturally in aquatic birds, may also affect other birds, lower mammals and humans [3]. The AIV belongs to the genus influenza virus A in the family Orthomyxoviridae [4]. That family shelters three influenza genera—A, B and C, however, only AIV type A (AIV-A) viruses infect birds [5]. The AIV-A is classified into subtypes based on two proteins on the surface of the virus: haemagglutinin (H) and neuraminidase (N) being known 18 HA and 11 NA subtypes [6].

All known subtypes of AIV-A can infect birds, but two AIV-A subtypes were discovered in bats (H17N10 and H18N11), and has only been found in bats expanding the host range of the virus [7-10]. However, the AIV infection among domestic poultry

Corresponding author: Maria Angela Orsi, Ph.D., research fields: avian disease and biologic quality control.

or confined bird may evolve a broad syndrome ranging from subclinical or mild upper respiratory disease to fatal disease depending on the virus virulence [11]. AI outbreaks reaching mortality level as high as 100% was associated with very virulent viruses named the highly pathogenic AI (HPAI). Consequently, the AI became one of the most relevant diseases for poultry industry worldwide [12]. Although, the HPAI have been restricted to subtypes H5 and H7, the occurrence of HPAI may exacerbated by co-infections or environmental conditions [13].

As the Egyptian pigeon simultaneous infection with HPAI subtype H5N1 and pigeon-paramyxovirus-1 (pPMV-1) was implicated with the large-scale HPAI outbreaks occurred between 2013 and 2015 [14]. It's important to point out that mainly after 1997, the AIV transmission from poultry to humans is causing severe clinical symptoms and death turned the AIV infection also a potential threat to human health [15].

The Laboratório Nacional Agropecuário de São Paulo-LANAGRO-SP or Agriculture and Livestock National Laboratory-São Paulo in the city Campinas, Brazil co-jointed with the National Veterinary Services Laboratories (NVSL) in Ames, Iowa, United State of America (USA), for the development of the Twinning Project (TP) under the rules of the World Organization for Animal Health (OIE).

The aim of TP was to establish an AIV-diagnostic international standard reagents production under rigorous quality control to attend the necessities of Brazil and other South American countries [16]. In this study, it was described systematically the production of AIV master seeds, inactivated antigens and hyperimmune serum following of the virus characterization in type and subtype to improving the laboratory diagnosis.

2. Materials and Methods

2.1 AIV Standard Samples

AIV standard samples denominated AIV original or

master seed s	stocks (N	188), can	he from N	/SL (Ames,
Iowa, USA)	through	the TP. T	The AIV or	iginal were:
NWS-NS-7/1	0/06			(H0N5),
A/WTFWL/C	GA/21823	36-37/07		(H1N1),
A/CK/NY/3750-7/76			(H2N2),	
A/Pintail/AL	BERTA/	293/77-7/	07/06	(H2N9),
A/DK/CZEH	/56			(H4N6),
A/TY/MN/-A	FF-10.2	3/03-NVS	SL	(H5N2),
A/TY/ONTA	RIO/63	(H6N8),	A/TY/UT/2	24721-10/97
(H7N3),	A/TY/O	NTARIO	/6118/67	(H8N4),
A/MAL/ALE	/187/83	(H9N1),	A/CK/FI	./374688/05
(H10N9),	A/DK/N	MEMPHI	S/546/74	(H11N9),
A/DK/ALBE	RTA/60/	76		(H12N5),
A/GULL/ME	0/704/779	9/81		(H13N6),
A/MAL/GUF	RJEV/263	3/82-A/BI	EL/42	(H14N1),
A/SHEARW	ATER/W	ESTERN	ſ	
AUSTRALIA	/2576/7	9	(H15N9)	and
A/SHOREBI	RDS/DE	/172/06 (1	H16N3).	

2.2 Animal Facilities and Biosafety Laboratory

MSS, the inactivated antigens, antiserum production specific pathogen-free (SPF) chickens assays and all samples processed were carried out in biosafety level 3 (BSL-3) containment of the Laboratório de Sanidade Aviária (Avian Disease Laboratory-ADL) from LANAGRO-SP.

2.3 AIV Protocols

The MSS, the inactivation of the infectious amniotic-allantoic fluid (AAF) and the antiserum production were following the protocols USDA [17-19], respectively.

2.4 Experimental Designs

2.4.1 AIV MSS and Antigens

The low-pathogenicity AIV MSS samples were diluted 10 fold in PBS. Then, 0.1 mL of the virus diluted 10^{-3} was inoculated into allantoic cavity in each of the 9-11 d old SPF embryonic chickens eggs (ECEs). The ECEs were incubated at 36-37 °C for 4 d and candled daily. The eggs with embryos dead in the

first day were discarded. The AAF collected individually onto four days post infection (DPI), tested by hemagglutination (HA) [17]. The HA-positive AAF were filtered and centrifuged. The AAF virus-fluid samples fragmented as master seed for stock and alive antigen for inactivation. Both were capping and labelling and conserved in the temperature -70 \pm 5 °C. In followed, the pool of antigen was submitted to HA and sterility tests. The fluids HA reactivity against one-panel AI sera H1-H16 and N1-N9 determined the AIV type specificity.

2.4.2 Antigens Inactivation

The AAF antigens previously tested for HA/AI placed in pre warms on room temperature until thawing, diluted at 1:38 of disodium phosphate solution (DSP) and treated with of β -propiolactone $(\beta$ -PL) dropwise with gently agitate the mixture until obtaining the final concentration 0.05% β-PL. The mixture has been homogenized in one rotator for 2 h, kept at 4 °C over night for finalizing the inactivation process. In following, the AIV inactivated antigen centrifuged at 1,500× g for 10 min, and diluted at 10^{-1} in phosphate buffer saline (PBS). Five embryonic eggs aging 9-11 d inoculated with 0.1 mL by the allantoic cavity route. The presupposed inactive antigen solution was passed in embryonic eggs twice for ensure virus inactivation. After the inactivation has been confirmed, the solution has stabilized on the pH 7.3-7.4 by applying 7.5% sodium bicarbonate solution. Then, centrifuged at 1,500× g for 20 min. Sodium azide was added to preserved antigen stock solution in final concentration of 0.1%. Then, the solution reagent lyophilized and conserved at -15 ± 5 °C. At end, the sterility level and final HA titer reached by the inactivated antigen were determined.

2.4.3 AIV Antiserum

2.4.3.1 Immunizations

Ten to thirty SPF White Leghorn chickens with 2-3 months of age, previously identified (ID) on the legs and maintained in isolators during the

experimental period of 14 d. The AIV master seed previously produced in the LANAGRO-SP (described in assay 1), AAF alive stored at -70 \pm 5 °C has thawed, clarified by centrifugation at 1,500× g for 30 min/4 °C. The SPF chicken with \leq 4.0 mL AAF, depending on age, size and sex were immunized by brachial vein route (wing vein). After 10 DPI, 2-3 mL of chicken's blood was collected by a wing vein and the serum sample tested hemagglutination inhibition (HI), individually. The birds were exsanguinated when with AIV antibody titer reaches equal or over 1:256 titer at 14 DPI. A booster immunization has been performed when the AIV antibody titer was below 1:256 at 14 DPI. The birds were re-exposed to the antigen-adjuvant systems (involving three antigens to seven of adjuvants) by subcutaneous route in three to four points with 0.25 mL dose. The immunogenicity enhancing has been realized at 10 DPI by the serum antibody re-tittered (Montanide ISA 206).

2.4.3.2 Avian Euthanized

Concern to cause minimum of pain and distress time each chicken before the killing, was inoculated with a combined mixture of sedative, muscle relaxant and anesthetics (5 mg/kg of xylasin hydrochloride and 50 mg/kg of ketamine hydrochloride) as recommended by Massone [20] and the Guidelines for Euthanasia of Animals [21]. On the 14 DPI, around \leq 1 d prior the exsanguination, the birds were off feed regimen.

2.4.3.3 Blood Collection

The exsanguination by cardiac puncture used a stainless steel needle attached to 60 cc syringes. Filling the syringe with 2/3 of full capacity was sufficient for a good separation of serum from the clot. Firstly, the blood samples were incubated at 37 °C \leq 2 h, and then at 22-25 °C by 18-24 h.

2.4.3.4 Serum Collection for Serology

The bird's sera collected in tubes of 25-50 mL, identified–labeled, centrifuged at $1,200 \times$ g during 15 min, decanted and stored in a new tube. Every serum

collected was submitted to the quality control.

2.5 Quality Control

2.5.1 Sterility Guarantee

The absences of viable microorganisms of the serum and antigens have carried out by conventional methods for microorganism growth. In blood agar, 0.1 mL of each serum has sown while 0.5 mL of each antigen was inoculated in sterile Brain heart infusion (BHI) broth, and incubated at 37 °C. The sterility was guaranteed because no microorganism has been growing in 2 d and 7 d for agar and BHI, respectively.

2.5.2 Titration

To determine the concentration of virus and specific antibodies each of antigens and serum samples were tittered in HA and HI methods, respectively. The similar titer in HA and HI test were pooling and stocked at -70 ± 5 °C and -15 ± 5 °C, respectively, and the pooling data registered at laboratory notebook.

2.5.3 Neuraminidase Antibody Specificity

This methodology may be performing for identification: AIV neuraminidase subtypes or neuraminidase antibody specificity. The AIV antigens were subtyping by the micro-neuraminidase-inhibition assay using N1 to N9 in accord with international and Brazilian rules [22, 23]. The neuraminidase inhibition test identified different AIV N subtypes. When the AIV and subtype-specific antibodies of reference antisera reacted, blocked the formation of a chromophore by the aldehyde NANA reaction with thiobarbituric acid, consequently no color development.

2.5.4 AIV H Subtypes Identification and Humoral Antibodies Quantification

Conventionally, the identification of the AIV H subtype antibodies specificity is carried on by HI test. The basis this methodology is inhibition of HA when a virus reacts with one subtype-specific antibody. The paramount was the confrontation with a library of reference (H1 to H16) reagents specifying AIV strain of a single H subtype among multiples lineages [24, 25], although it involves an extensive laboratory support for the reagents production and optimization.

2.6 Statistical Analysis

The correlation between the quantity of antigens used in the process of immunization and the HI antibody titer obtained were evaluated by the Mantel Haenszel chi-square test [26].

3. Results

3.1 AIV Master Seed

The production of low-pathogenicity AIV original seed in eggs was successful. The antigenic proteins haemagglutinin: H0, H1, H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16 and neuraminidase N1, N2, N3, N4, N5, N6, N8 and N9, were determined. Seventeen (17) AI master seed (antigens) subtypes produced lots reached HA titers ranging 1:512 to 1:32 (Table 1).

3.2 Antisera

The HI/AI antibody titer of 15 antiserum lots ranged from 1:4,096 to 1:256. The AIV subtypes based on HA and NA antigenic protein were H1 to H16, except the H12, and N1, N2, N3, N4, N6, N7, N8 and N9 except N5, respectively. The best HI titer was 1:4,096 for the subtype H1N7. The AIV antibody titer begun to decline on the 1:2 scale down lessening 1:2,048 in H2N9, H4N6 and H5N2; 1:1,024 for H3N1, H7N1 and H13N6; 1:512 for H10N9 and H14N1 and 1:256 for H6N8, H8N4, H9N1, H11N9, H15N9 and H16N3 subtypes. To improve the antibody titers of H1N7, H2N9, H4N6, H9N1 and H16N3 subtypes an AIV booster, in the same previous conditions, was performed.

The quantity of different subtypes of AIV antigens were inoculated in female = F (2.0-4.0 mL) and male = M (2.5-4.0 mL) during the immunization process. It was observed that the antigen H11N9 using the dose of 2.0 mL F and 3.0 mL M can be able to produce

		······
Subtype name	Virus strain	Titer (HA)
H0N5	NWS-NS-7/10/06	1:512
H1N1	A/WTFWL/GA/218236-37/07	1:256
H2N2	A/CK/NY/3750-7/76	1:32
H2N9	A/PINTAIL/ALBERTA/293/77	1:256
H4N6	A/DK/CZEH/56	1:512
H5N2	A/TY/MN/- AFF-10/23/03	1:512
H6N8	A/TY/ONTARIO/63	1:256
H7N3	A/TY/UT/24721-10/97	1:128
H8N4	A/TY/ONTARIO/6118/67	1:256
H9N1	A/MAL/ALB/187/83	1:64
H10N9	A/CK/FL/374688/05	1:256
H11N9	A/DK/MEMPHIS/546/74	1:256
H12N5	A/DK/ALBERTA/60/76	1:256
H13N6	A/GULL/MD/704/779/81	1:512
H14N1	A/MAL/GURJEV/263/82-A/BEL/42	1:256
H15N9	A/SHEARWATER/WESTERN AUSTRALIA/2576/79	1:256
H16N3	A/SHOREBIRDS/DE/172/06	1:64

Table 1 Avian influenza (AI) master seed subtypes production, virus strain and hemagglutination (HA) titer produced.

Seventeen (17) AI master seed (antigens) subtypes produced, the virus strains used in production and the lots reached HA titers ranging 1:512 to 1:32.

antibodies with the HI titer of 2⁻⁸. The antigen H4N6 using the dose of 2.0 mL F and 3.5 mL M can be unable to produce antibodies with HI titer $\geq 1:256$ being necessary to make booster immunization, and the final HI titer was 2^{-11} . The antigens used the dose 2.5 mL in F and M, and the quantity of antibodies produced was for H10N9 2⁻⁹ and to H3N1 was 2⁻¹⁰. When the doses used were as 2.5 mL F and 3.0 mL M the antigen H13N6 could be able to produce the quantity of antibodies 2⁻¹⁰. However, the H1N7 was unable to be produced being necessary booster and the HI titer final was 2^{-12} . When the antigen doses used was 3.0 mL for F and M the subtype H8N4 was able to produce HI titer of the 2⁻⁸ but the antigens H9N1 and H16N2 was unable to produce HI titer 2⁻⁸, being necessary to make booster to get titer. The antigens H6N8 using the 3.0 mL in F and 3.5 mL in M can be able to produce the quantity of the antibodies of 2^{-8} . The doses 3.5 mL in F and M with the following antigens H14N1 got the HI titer 2⁻⁹, the H7N1 got the HI titer of the 2⁻¹⁰ and for the subtype H2N9 was necessary booster immunization to reaching HI titer of the 2⁻¹¹. The antigen H15N9 used the doses 3.5 mL F and 4.0 mL for production of antibodies and the HI titer obtained was 2^{-8} , however when the dose 4.0 mL for F and M was used for H5N2 the HI titer was 2^{-11} .

All antisera data submitted to Mantel-Haenszel chi-square test to measuring the correlation among the quantities of antigens inoculated in each Gallus and HI titer attained. However, did not figure out a correlation about them (p = 0.8882), suggesting the titers are independent of the amounts of antigen used (Table 2).

3.3 AIV Master Seed before and after Inactivation

The AIV HA titer comparison between AIV master seed before and after inactivation process demonstrated a descending titer scale. The following H2N9, H7N3, H11N9, H15N9 and H16N3 subtype viruses maintained the same original HA titer, representing the rate of 5/14 (35.71%). In following, the titer of inactivated H8N4 and H9N1 virus subtypes or 2/14 (14.28%) dropped to 1log2. Whilst the titer differences for H1N1, H5N2, H6N8 and H12N5 virus subtypes or 4/14 (28.57%) dropped 2log2.

The titer of inactivated virus still moving more downwards, H10N9 subtype viruses or 1/14 (7.14%)



Fig. 1 Comparison of hemagglutination (HA) titers between AIV master seed (0 h) and with β-propiolactone (β-PL) inactivated virus (18 h): (a) H1N1, H2N9, H5N2, H6N8, H7N3, H8N4 and H9N1 subtypes; (b) H10N9, H11N9, H12N5, H13N6, H14N1, H15N9 and H16N3 subtypes.

Table 2 Immune response induced by AI virus (AIV).

Statistic test	df	Value	Prob (<i>p</i>)
Mantel-Haenszel chi-square	1	0.0198	0.8882

The AIV chicken-inoculated and the immune answers elicited did not figure out in one correlation among them, independent of the amounts of antigen used.

has fallen 3log2, and H13N6 and H14N1 subtypes or 2/14 (14.28%), drooped down 4log2 in the HA/AI titer (Figs. 1a and 1b).

In immunization chickens, the comparison of AIV antigen titration before (HA titter) and after of immunization (HI titer) showed the 100% titer concordance to three (30%) H6N8, H8N4 and H15N9 subtypes. At the meantime, a significant increasing of the antibodies titer against AIV elicited by immunization has been observed in 70% animals. The AIV antibody titer (HI) increased 1log2 for three subtypes (H10N9, H13N6 and H14N1) (30%). The animals immunized the H5N2, H9N1 and H16N3 subtypes (30% again) the HI titer growth 2log2.

Highlighting, although it was from one AIV H2N9 subtyped, the antibody titer elicited by immunization growth 3log2 (Fig. 2).

3.4 AIV Inactivated Antigen Titration

Seventeen (17) lots of inactivated antigens with the titer HA ranging from 1:256 to 1:16, encompassed H1 to H16 subtypes, except the H4 and N1 to N9 subtype, except N7. In the AIV antigen inactivation assay, the highest HA titer and the best result achievement were for H2N9, H11N9 and H15N9 (1:256). In following down H3N1, H3N8, H5N2, H7N3 and H8N4 (1:128). The HA titer decreased more to H1N1, H6N8, H7N1, H12N5 and H16N3 (1:64) and for H9N1, H10N9 and



Fig. 2 Comparison of the HA titer (HA/AI) of the master seed antigen used in immunization process (black figure) and the HI titer of antiserum produced (scorched figure).

Antigen name	HA titer
H1N1	1:64
H2N9	1:256
H3N1	1:128
H3N8	1:128
H5N2	1:128
H6N8	1:64
H7N1	1:64
H7N3	1:128
H8N4	1:128
H9N1	1:32
H10N9	1:32
H11N9	1:256
H12N5	1:64
H13N6	1:32
H14N1	1:16
H15N9	1:256
H16N3	1:64

 Table 3 AIV subtypes inactivated antigens titrated by HA test (HA titer) as recommended international standard methods.

Seventeen (17) AIV subtypes inactivated antigens produced, from H1 to H16 except H4 and lots reached HA titers ranging from

1:256 to 1:16.

H13N6 (1:32). The smallest HA/AI titer (1:16) was for H14N1 subtype (Table 3).

4. Discussion

The Asian human influenza outbreaks in 1997 caused by the AIV triggered the development of several protocols to AIV diagnosis. The AIV published protocols for laboratory diagnose tests are quite similar, conventionally performed to characterize the virus by isolation in eggs followed the HA and HI to subtypes identification and genomic analysis [27]. The AIV becomes a threatening to human health worldwide by the ability to cross the borders through infected migratory birds or human translocation highlighted the necessity of an international standard for the virus laboratory diagnosis [28].

In 2006 and 2007, the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) has presented the national prevention plan for AI conjoined with control and prevention of Newcastle disease, and the contingence plan for AI and Newcastle disease, respectively. Both plan intended to improve AIV and Newcastle disease virus (NDV) laboratory methods in accordance with OIE (World Organization for Animal Health) standards to carry out defensive actions against HPAI and NDV. The primordial purpose was to produce the high quality diagnostic standard reagents under OIE expertise assistance. All production procedures have performed under biosafety facilities to avoid the virus dissemination for environment.

In this work, the master seed production has been performed in SPF-ECE to applying in diagnostic routine. The concern to the AIV genetic variation, the MSS production included 17 AIV samples isolated in a poultry chickens and wild birds.

The best results of HA MSS titer was 1:512 for subtypes H0N5, H4N6, H5N2 and H13N6. The HA titer was 1:256 to the following subtypes: H1N1, H2N9, H6N8, H8N4, H10N9, H11N9, H12N5, H14N1 and H15N9. To the H7N3 subtype, the titer was 1:128. The H9N1 and H16N3 subtypes had the HA titer of 1:64. The subtype H2N2 reached 1:32, the lowest HA titer. Although, it was not for vaccine production, the entire master seed produced have to attend quality standards exigencies for sterility, purity and safety [12, 27, 29].

In 1989, the high potential of toxicity and carcinogenesis were demonstrate when β -PL have been induced rat nasal carcinoma by the activation of a new oncogene however, it had occurred when the mouse were directly submitted to inhalation or by topical administration [30]. The β -PL has been strengthening potential to AIV inactivation consequently the potential hazard of poultry contamination with AIV avirulent strain avoided when the virus samples were inactivated by β -PL previously. The β-PL treatment, has been successfully applied for a long time in the production of several virus-inactivated vaccines and for virus suspensions inactivation because it is able to modify chemically the building blocks of nucleic acids and proteins [31]. Structurally, β -PL is an organic compound consisting of almost planar highly strained four-membered ring included in the lactone family with high energetic eletrophilicity [31] that in aqueous solutions made it be able to react with nucleophiles forming conjugates or adducts by covalent bonds with target molecules, such as protein or DNA [32]. Uittenbogaard et al. [31] suggested the occurrence of at least one modification induced by the treatment with β -PL in nucleic acid either deoxyguanosine, deoxyadenosine or cytidine although, no reaction products were found from thymidine and uracil. However, the β -PL affects more intensively virus proteins reacting with nucleophilic group (1a or 2a amines) and possibly blocking the viral infectivity. Bonnafous et al. [33] suggested the loss of influenza virus infectivity submitted to β -PL treatment was dose-dependent after assayed the H3N2 strain to β -PL in various concentrations ranging 2-1,000 µM. The virus infectivity put an end at 1 mM β -PL. A matter of fact, the epithelial cells of the respiratory tract is the main targets of the influenza virus. Even if, the surface differences of HA among AIV and humans and aquatic birds influenza virus [34], the AIV bulge glycoprotein haemagglutinin binding to sialic acid moieties on the surface of host cells and triggers the membrane fusion [35]. Therefore, the fusion protein is necessary for the effective infection and a possible target for virus inactivation.

To determine the potency of inactivated virus for HI test, the comparison of the titers of inactivated and live virus was performed. The titration assays have to achieve at least two goals: (1) to increase of titer of master seed antigens; (2) optimized the inactivation process in order to avoid a lower loss of titer in this process. The HA titer the master-seed produced ranging from 1:512 to 1:32 and the titer of the antigens inactivated from 1:256 to 1:16. Those results showed one loss of $\leq 1\log 2$ in 50% dos antigens live inactivated. Unfortunately, only 2/14 subtypes produced have similar results was obtained by Jonges et al. [36], whom reached a rate at least of 10 fold in the reduction of HA titer and NA activity of the human influenza virus applying β -PL. These results suggested the higher virus titer might be necessary in the beginning of inactivation process to compensate this loss. The β -PL inactivation mechanism remains poorly understood and reactions not predicted may occur. In contrast, for vaccine production, the diminishing of the HA titer, antigenicity and NA activity of the human influenza virus may compromise the vaccine potency [37].

After the emergence of AIV in humans, the necessity to knowledge the virus epidemiology and ecology focusing the virus transmission from birds to birds to human and the risk factors associated with infection [38, 39]. However, the poultry AIV infection may occur by very virulent viruses causing the HPAI principally by H5 and H7 subtypes or by low pathogenic AI (LPAI), causing milder disease

that can exacerbated by other infections or environmental conditions [40]. In birds the AIV sera-surveillance assays is most common to detect antibody against to LPAI than HPAI [28, 35]. Particularly in wild birds including geese, shorebirds and wild ducks, been the last one considered the natural reservoirs of influenza A viruses [38-40]. In the natural AIV infection, the birds prompt a humoral and mucosal antibody immune response [41]. Then, the AIV sera surveillance assays is necessary and it is widely performed by HI technique to identify the AIV into subtypes based on the sera-groupings of 16 haemagglutinin and nine neuraminidase [42].

By the way, Suarez and Schultz [41] asserted AIV poultry infected naturally elicit the humoral immune response systemic as well as mucosal antibody. In both chickens and turkey AIV infected the immunoglobulin M (IgM) and immunoglobulin Y (IgY) are detected as 5 DPI and shortly after, respectively. Then, similarly with other species the antibodies screen have to perform as early as 7 d after infection [43]. The elicited of antibody by chickens AIV infected may correlated with the antigens titer inoculated. The level of humoral antibodies measured by HI titer after 10 DPI and 14 DPI was supported by NVSL/USDA protocols. The antibody titer elicited in 1/3 of immunized birds reached \geq 1:256 at 10 DPI but, at 14 DPI, in all immunized group sera the titer were similar $\geq 1:256$ or greater than 1log2 in the HI/AI test.

Yang *et al.* [44] applying a competitive ELISA (c-ELISA) showed that chickens experimentally infected with the H5N3 the NP antibodies NP were measure on 7 DPI, but the antibodies elicited by homolog antigens, the positive HI response occurred in 14 DPI. It may occurred because the NP protein could be prior expression in HA test [45].

The quality control of the immune reagents were based the OIE/WHO standards methodologies [12, 24, 27].

5. Conclusions

The necessity of AIV diagnosis to identify endemic, epidemic and seasonal influenza strains and other wildlife influenza subtypes that cross the country's borders elicited the development of international standards. The Twinning Project (TP) with OIE resulted in production of AIV-master seeds, inactivated antigens and antiserum to attend the necessities of Brazil and other South America countries. The 17 AIV reference propagating to production the best quality master seed and then, the AIV antigen inactivation; the H and N AIV subtypification demonstrated the LANAGRO team is able to produce specificity and sensitivity standard reagents. Although, the titer of the AIV master seeds produced should be increased to be better, all the influenza reference reagents were successfully produced.

Acknowledgments

The authors are grateful to Luiz Carlos S. Porto, Adilson R. Belarmino and Manuel C. Gonçalves de Aguiar for technical assistance.

References

- ABPA. 2017. Brazilian Association of Animal Protein Annual Report. Accessed March 12, 2018. http://abpa-br.com.br/storage/setores/avicultura/publicaço es/relatorios-anuais. (in Portuguese)
- [2] Brazil. 2006. "Contingency Plan for Avian Influenza and Newcastle Disease." Normative Instruction n. 17 of April 07, 2006. Secretariat of Agricultural Defense, MAPA. Official Journal of the Union, Issue 1, Number 69. (in Portuguese)
- [3] Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. 1992. "Evolution and Ecology of Influenza A Viruses." *Microbiol. Reviews* 56 (1): 152-79.
- [4] International Committee on Taxonomy of Viruses (ICTV). 2015. Virus Taxonomy. Accessed June 20, 2016. http://www.ictvonline.org/virus Taxonomy.asp.
- [5] Rahman, M. S., Rabbani, M. G., Uddin, M. J., Chakrabartty, A., and Her, M. 2012. "Prevalence of Avian Influenza and Newcastle Disease Viruses in Poultry in Selected Areas of Bangladesh Using Rapid Antigen Detection Kit." Archives of Clinical

Microbiology 3 (1:3): 1-8. doi:10.3823/248.

- [6] CDC. 2017. "Influenza Virus Subtypes." Accessed Mar. 13, 2018. https://www.cdc.gov/flu/avianflu/influenza-a-virus-subty pes.htm.
- [7] Mehle, A. 2014. "Unusual Influenza A Virus in Bats." Viruses 6 (9): 3438-49.
- [8] Worobey, M., Han, G. Z., and Rambaut, A. 2014. "A Synchronized Global Sweep of the Internal Genes of Modern Avian Influenza Virus." *Nature* 508 (7495): 254-7.
- [9] Swayne, D. E., Suarez, D. L., and Sims, L. D. 2013. "Influenza." In *Diseases of Poultry*, 13th ed., edited by Swayne, D. E., Glisson, J. R., McDougald, L. R., Nair, V., Nolan, L. K., and Suarez, D. L. Ames, Iowa, USA: Wiley-Blackwell, 181-218.
- [10] Tong, S., Li, Y., Rivailler, P., Conrardy, C., Alvarez, D., Chen, L. M., Recuenco, S., Ellison, J. A., Davis, C. T., York, I. A., Gilbert, A. T., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M. R., Tang, K., Rowe, L. A., Sammons, S., Xu, X., Frace, M., Lindblade, K. A., Cox, N. J., Anderson, L. J., Rupprecht, C. E., and Donis, R. 2012. "A Distinct Lineage of Influenza A Virus from Bats." *Proc. Natl. Acad. Sci.* 109 (11): 4269-74.
- [11] Lyra, T. 2015. "Avian Influenza." CNA, Brazil, 1-6. (in Portuguese)
- [12] World Organization for Animal Health (OIE). 2015. "Chapter 2.3.4 Avian Influenza (Infection with Avian Influenza Viruses)." in *Terrestrial Manual*, 1-23.
- [13] Alexander, D. J. 2000. "A Review of Avian Influenza in Different Bird Species." *Vet. Microbiol.* 74 (1): 3-13.
- [14] Mansour, S. M. G., Mohamed, F. F., Eid, A. A. M., Mor, S. K., and Goyal, S. M. 2017. "Co-circulation of Paramyxo- and Influenza Viruses in Pigeons in Egypt." *Avian Pathol.* 46 (4): 367-75.
- [15] Perdue, M. L., and Swayne, D. E. 2005. "Public Health Risk from Avian Influenza Viruses." Avian Dis. 49 (3): 317-27.
- [16] Brazil. MAPA. 2011. Final OIE Twinning Report, 1-27.
- [17] USDA, CVB and NVSL (AVPRO0801.01). 2004. Preparation of Avian, Equine, and Swine Influenza (Ortomyxovirus) Hemagglutinating Antigens, USDA, 1-10.
- [18] USDA, CVB and NVSL (AVSOP2226.02). 2003a. Beta-propiolactone Inactivation of Orthomyxoviruses and Paramyxoviruses, 1-6.
- [19] USDA, CVB and NVSL (SOP-AV-0058.01). 2009. Preparation of Control Antisera for the Avian Paramyxovirus and Influenza A Virus Hemagglutination Inhibition Tests, 1-9.
- [20] Massone, F. 2003. Veterinary Anesthesia-Pharmacology and Techniques. Guanabara Koogan Pres., 4th edition, 344.

Development of Standard Reagents for Avian Influenza Virus Subtypes Diagnosis

- [21] Leary, S., Underwoo, W., Raymond A., Cartner, S., Corey, D., Walla, W., Greenacre, C., Lead, N., McCracki, M. A., Meyer, V. R., Miller, D., Shearer, J., Yanong, R., Golab, G. C., and Patterson-Kane, E. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. American Veterinary Medical Association. ISBN 978-1-882691-21-0.
- [22] USDA, CVB and NVSL (AVPRO1300.03). 2011. Micro Neuraminidase-Inhibition Procedure for Subtype Identification of Influenza A Virus Neuraminidase, 1-13.
- [23] Brazil, LANAGRO/SP (Met AVI_041_002). 2016. Inhibition of Neuraminidase for the Subtyping of Influenza A Virus, 1-6. (in Portuguese)
- [24] Pedersen, J. C. 2014. "Hemagglutination-Inhibition Assay for Influenza Virus Subtypes Identification and the Detection and Quantitation of the Serum Antibodies to Influenza Virus HI Assay." *Methods Mol. Biol.* 1161: 11-25.
- [25] Brazil, LANAGRO/SP (Met AVI/007/005). 2017. Inhibition of Hemagglutination for the Detection of Antibodies to Newcastle Disease Virus and for Subtyping of Antibodies to Influenza Virus A, 1-7. (in Portuguese)
- [26] Viali, L. 2008. "Non-Parametric Tests." Porto Alegre, RS,
 32. Accessed May 29, 2018. http://www.mat.ufrgs.br/~viali/estatistica/mat2282/materi al/laminaspi/Mat2282_2_Indep.pdf. (in Portuguese)
- [27] World Health Organization (WHO). 2007. Guidelines on Laboratory Diagnosis of Avian Influenza. World Health Organization 2007, Regional Office for South-East Asia. SEA/CD/171, 1-21. http://www.who.int/iris/handle/10665/205182.
- [28] Chung, P. H., Mumford, L., Perdue, M., Prosenc, K., Zambon, M., Peiris, M., Perkins, M., Wood, J., Alexander, K., Meijer, A., and Jennings, L. 2007. "Expert Consultation on Diagnosis of H5N1 Avian Influenza Infections in Humans." *Influenza and Other Respir Viruses* 1 (4): 131-8.
- [29] Minor, P. D., Engelhard, O. G., Wood, J. M., Robertson, J. S., Blayer, S., Colegate, T., Fabry, L., Heldens, J. G., Kino, Y., Kistner, O., Kompier, R., Makizumi, K., Medema, J., Mimori, S., Ryan, D., Schwartz, R., Smith, J. S., Sugawara, K., Trusheim, H., Tsai, T. F., and Krause, R. 2009. "Current Challenges in Implementing Cell-Derived Influenza Vaccines: Implications for Production and Regulation, July 2007, NIBSC, Potters Bar, UK." Vaccine 27 (22): 2907-13.
- [30] Garte, S. J., and Hochwalt, A. E. 1989. "Oncogene Activation in Experimental Carcinogenesis: The Role of Carcinogen and Tissue Specificity." *Environ. Health Perspect.* 81: 29-31.
- [31] Uittenbogaard, J. P., Zomer, B., Hoogerhout, P., and Metz, B. 2011. "Reactions of Beta-propiolactone with

Nucleobase Analogues, Nucleosides and Peptides: Implications for the Inactivation of Viruses." *J. Biol. Chem.* 286 (42): 36198-214.

- [32] Sanders, B., Koldijk, M., and Schuitemaker, H. 2015.
 "Chapter 2: Inactivated Viral Vaccines." In *Vaccine Analysis: Strategies, Principles, and Control,* Nunnally, B. K., Turula, V. E., and Sitrin, R. D. (eds.), 45-80.
- [33] Bonnafous, P., Nicolaï, M. C., Taveau, J. C., Chevalier, M., Barrière, F., Medina, J., Bihan, O. L., Adam, O., Ronzon, F., and Lambert, O. 2014. "Treatment of Influenza Virus with Beta-propiolactone Alters Viral Membrane Fusion." *Biochim et Biophys Acta* 1838 (1): 355-63.
- [34] Matrosovich, M., Zhou, N., Kawaoka, Y., and Webster, R. 1999. "The Surface Glycoproteins of H5 Influenza Viruses Isolated from Humans, Chickens, and Wild Aquatic Birds Have Distinguishable Properties." J. Virol. 73 (2): 1146-55.
- [35] Byrd-Leotis, L., Cummings, R. D., and Steinhauer, D. A. 2017. "The Interplay between the Host Receptor and Influenza Virus Hemagglutinin and Neuraminidase." *Int. J. Mol. Sci.* 18 (7): 1541.
- [36] Jonges, M., Liu, W. M., van der Vries, E., Jacobi, R., Pronk, I., Boog, C., Koopmans, M., Meijer, A., and Soethout, E. 2010. "Influenza Virus Inactivation for Studies of Antigenicity and Phenotypic Neuraminidase Inhibitor Resistance Profiling." J. Clin. Microbiol. 48 (3): 928-40.
- [37] She, Y.-M., Cheng, K., Farnsworth, A., Li, X., and Cyr, T. D. 2013. "Surface Modifications of Influenza Proteins upon Virus Inactivation by β-propiolactone." *Proteomics* 13 (23-24): 3537-47.
- [38] Ferro, P. J., El-Attrache, J., Fang, X., Rollo, S. N., Jester, A., Merendino, T., Peterson, M. J., and Lupiani, B. 2008.
 "Avian Influenza Surveillance in Hunter-Harvested Waterfowl from the Gulf Coast of Texas (November 2005-January 2006)." J. Wildlife Dis. 44 (2): 434-9.
- [39] Capua, I., and Alexander, D. J. 2007. "Avian Influenza Infections in Birds: A Moving Target." *Influenza Other Respir Viruses* 1 (1): 11-8.
- [40] Pawar, S. D., Kale, S. D., Rawankar, A. S., Koratkar, S. S., Raut, C. G., Pande, S. A., Mullick, J., and Mishra, A. C. 2012. "Avian Influenza Surveillance Reveals Presence of Low Pathogenic Avian Influenza Viruses in Poultry during 2009-2011 in the West Bengal State, India." *Virol J.* 9: 151.
- [41] Suarez, D. L., and Schultz, C. S. 2000. "Immunology and Avian Influenza Virus: A Review." *Dev. Comp. Immunol.* 24 (2-3): 269-83.
- [42] Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A., Gilbert, M., Nielen, M.,

and Stegeman, A. 2005. "Highly Pathogenic Avian Influenza H5N1, Thailand, 2004." *Emerg. Infect Dis.* 11 (11): 1664-72.

- [43] Swayne, D. E., and Halvorson, D. A. 2003. Influenza in Diseases of Poultry, 11th ed. Ames: Iowa State Press, 135-60.
- [44] Yang, M., Clavijo, A., Pasick, J., Salo, T., Wang, Z., Zhao, Y., Zheng, D., and Berhane, Y. 2011. "Serologic

Detection of Avian Influenza H5 Antibodies Using a Competitive Enzyme-Linked Immunosorbent Assay (ELISA)." J. Vet. Med. and Animal Health 3 (3): 56-61.

 [45] Prokudina, F. N., and Semenova, N. P. 1991.
 "Localization of the Influenza Virus Nucleoprotein: Cell-Associated and Extracellular Non-virion Forms." J. Gen. Virol. 72: 1699-702.