SEROLOGIC STUDIES OF SWINE INFLUENZA INFECTION IN ALBANIA

VALENTIN SHTJEFNI[1*], ILIRIAN KUMBE[2], DOMENICO BUONAVOGLIA[3]

[1]Department of animal health, Institute of Food Safety and Veterinary, Tirana, Albania

[2] Agricultural University of Tirana, Faculty of Veterinary Medicine, Tirana Albania

[3] Universita degli studi "Aldo Moro, Bari, Italia

* Author of correspondence; Email: valentine_shtjefni@hotmail.com

Abstract:

Three subtypes, H1N1, H1N2 and H3N2 influenza A viruses, are currently co-circulating in the swine populations in Europe. This study reports the current seroprevalence of swine H1 and H3 influenza in the swine populations in Albania by ELISA assay according to age group, management and rigging. This study extends in a three year investigation of antibody prevalence against SIV in swine serum samples collected from 16 regions of Albania. Blood samples from both intensive, and extensive swine farms were the sera used to perform the test. The prevalence of seropositivity against SIV during 2007-08 was 19.3%. During 2008-09, the prevalence of seropositivity against swine H1 virus was 12.2% and the swine H3 virus was 8.9%, and 2.6% were suspected cases. The cross reactivity was 5.7%. During 2009-10, the prevalence of seropositivity against the swine H1 virus was 18,3%, the swine H3 virus was 1,6%, and 1,2% were suspected cases. The cross reactivity was 1,2%. The highest prevalence of seropositivity was detected in the 3-6 months age group (11.3%), meanwhile the prevalence of seropositivity in intensive farms was higher than in extensive farms (12.7%).

Keyword: Antibody, Elisa, influenza, swine, virus.

1. Introduction

Influenza, a viral infection of the respiratory system spread world - wide in humans as well as animals, is caused by RNA viruses of the family Orthomyxoviridae. Each host is usually infected by specific strains, but interspecies transmission sometimes occurs. On the basis of antigenic properties of their nucleoprotein, these viruses are identified as types A, B or C. Type A viruses, which have mainly been isolated from farm animals, are further classified according to their surface antigens termed haemagglutinin and neuramidase [9]. The influenza viruses most commonly isolated from pigs all over the world are those bearing either H1N1 or H3N2 surface antigens or, occasionally, their combination. Three different HA/NA configurations or subtypes of swine influenza, i.e., H1N1, H3N2 and H1N2, have so far been isolated in Europe [7]. The classical H1N1 subtype is widely spread mainly in the USA and Asia. In Europe it was prevailing in the 1950s and 1960s, and was subsequently replaced by an avian variant of the H1N1 subtype transmitted from wild birds [1]. This variant, gradually became established instead of the original H1N1 subtype. The H3N2 subtype of influenza virus was first isolated from pigs in 1969 after the 1968 human pandemic [6], since then, at irregular intervals, it has been isolated from large pig herds all over Europe and, most recently, also on the American Continent [5]. The H1N2 subtype, since 1994 isolated in France [3], Great Britain [2] and Japan, [4], has probably arisen by reassortment of the human H1N1 and swine H3N2 viruses. Although swine influenza is, at present, a rare infection in the Albania, in the 1980's it accounted for moderate losses in large pig herds The first isolation of swine influenza virus was reported in 1980 [8]. The lack of information on the epidemiological situation in our pig herds was the reason for a survey of the current status of swine influenza in Albania. The results of the prevalence of SIV Ab subtypes in twenty two herds are reported in this study.

2. Material e Methods

2.1. *Manner of drawing blood :*

For proper performance of serologic test the blood serum must be obtained properly. Blood samples of pigs mainly were collected through the slaughtering process (free flow from stabbing ventral neck) and in a small part of them were obtained through sequential bleeding (from marginal ear veins). Samples are collected late in autumn to the whole winter from non vaccinated animals. Blood samples were labeled to identify each sample, and include the date and time that the sample was taken, are kept chilled (3–10°C), and then are transported to the testing laboratory as quickly as possible. Collected

blood samples are permitted to clot in sterile flacons or tubes. The clot is separate from the test flacon (tube) wall and serum left at refrigerator temperature for many hours or overnight to permit clot restriction. The flacon (tube) is then centrifuged and the clear serum free from hemolysis, is transferred by pipette into another tightly stoppered sterile tube. Samples of sera were stored at – 20 ^{0} C prior to testing. We chose the time period of this study to correspond with the seasonality of swine influenza.

2.1 Serologic examination for swine serum antibody.

ELISA kit CIVTEST SUIS INFLUENZA HIPRA Laboratories. Type of assay: Indirect ELISA. Utility: Diagnoses of Porcine flu Type A.

IDEXX HerdChek. Swine Influenza Virus Antibody Test Kit-H1N1 & Swine Influenza Virus Antibody Test Kit-H3N2

Enzyme – linked immunosorbent assay. Swine serum samples for detection of SIV antibody were tested from commercially different ELISA Test Kits. The first commercial ELISA kit used to assess SIV antibody response was not intended to differentiate between subtype – specific influenza virus exposure. Principle of the test: The specific antigen of Swine Influenza Virus (SIV) Type A is coated on well plate. Upon incubation of the sample (sera to be tested) in the test well, antibodies specific to SIV binds to the coated antigen and remains in the well after washing of the unbound material, then a conjugated is added that binds any attached swine antibody. After that, unbound conjugate is washed away and peroxidase-specific chromogenic substrate is added.

Table 1. Presence of antibodies against SIV type A in different age group in Albania (2007-08)

Age Group	**Nr of sera Herd/individual pig**	**Nr (%) of positive sera (Herd individual pig)**	**Nr (%) of negative sera (Herd individual pig)**
		SIV Antibodies	
3-6 months	21 237	11(52.3) 75(31,6)	10(47,6) 162 (68,4)
6-12 months	21 221	6 (28,6) 14 (6,3)	15 (71.4) 207 (93.7)
> 12 months (Sows & Boars)	21 2	0 0	21(100) 2 (100)
Total	21 460	11 (52.4) 89 (19.3)	10 (47.6) 371(80.7)

Table 2. Presence of antibodies against SIV in different age group in Albania (2008-09)

Age Group	**Nr of zero Herd/individual pig**	**Nr (%) of positive sera (herd/individual pig)**			
		H1N1	**H3N2**		
			Positive	**Suspected**	**Cross reactivity**
3-6 months	22 207	8 (36.4) 10 (4.8)	9 (40.9) 22 (10.6)	6 (27.3) 6 (2.9)	3 (13.6) 5 (2.4)
6-12 months	22 193	6 (27.3) 37 (19,1)	8 (36.4) 18 (9.3)	6 (27.3) 6 (3.1)	6 (27.3) 19 (9.8)
> 12 months (Sows & Boars)	22 59	8 (36.4) 9 (15.2)	1(4.5) 1 (1.7)	0 0	2 (9) 2 (3.4)
Total	22 459	8 (36.4) 56 (12.2)	9 (40.9) 41 (8.9)	6 (27.3) 12 (2.6)	6 (27.3) 26 (5.6)

Essentially : Samples to be tested were diluted 1: 200 in following the 2 step dilution protocol. Sample diluent solution (provided in the kit) were 3 (x) concentrated and for its reconstruction we added 1 volume of the sample diluent solution to 2 volumes of distilled water (e.g. To prepare 60 ml reconstituted solution we mixed 20 ml of the concentrate solution with 40 ml distilled water). In the first step 5µl of sample were diluted in 95µl of reconstituted sample diluent solution pipetting each sample in a dilution plate, and in the second step we transferred 5µl of this 1: 20 diluted sample to the ELISA well which has previously been pipetted 45µl of the sample diluent solution. Fifty micro liters of each control and diluted samples were transferred to the appropriate wells and incubated for 60 min at 37^{0}C. After incubation, the controls and samples were discarded from wells, and plates were washed with a reconstituted washing solution (300 µl per well). Fifty micro liters of peroxidase-labeled anti-IgG secondary antibody, were

dispensed to each well and incubated for another 60 min. After rinsing, the presence of antigen–antibody complexes was observed by adding 50 µl of substrate to each well. Optical density (OD) of each well was measured using a micro titration plate reader at 405 nm. For interpretation of results the specific formula is applied to obtain the IRPC (Relative Index x 100) value. Samples with IRPC value > 20 were considered to be positive, whereas samples with IRPC value ≤ 20 were considered to be negative. The prevalence of seropositivity against SIV during 2006 - 07 was 19.3% (89/460).

In the second and third cases we have used an ELISA kit that was developed by IDEXX Laboratories for the preferential detection of antibodies against swine influenza virus subtype H1N1 and H3N2. Enzyme–linked immunosorbent assay was performed according to the protocol recommended by the producer. All reagents were allowed to reach room temperature (20 ^{0} C–25 ^{0} C) before use. Samples to be tested were diluted 1:40 in sample diluents provided in the kit (10µl serum added to 390µl sample diluents). One-hundred micro liters of each control and diluted sample were transferred to a well and incubated for 30 min. After incubation, the controls and samples were discarded from wells, and plates were washed using multi-channel pipettes. One-hundred micro liters of peroxidase-labeled anti-IgG secondary antibody, which was also provided in the kit, were dispensed to each well and incubated for another 30 min. After rinsing, the presence of antigen–antibody complexes was observed by adding 100 µl of substrate to each well. Optical density (OD) of each well was measured using a micro titration plate reader at 650 nm. OD values were then converted into sample-to-positive (S/P) ratios using a formula provided by the manufacturer. Samples with S/P ratio equal to or greater than 0.4 were considered to be positive for antibody against H1N1 SIV. The ELISA test for detection of H3N2 antibodies was performed in the same manner as described above with one exception, interpretation of S/P ratio. In this case : samples resulting in a S/P ratio < 0.30 are considered negative for antibodies to swine influenza virus (H3N2), samples resulting in a S/P ratio ≥ 0.30 and < 0.40 are classified as suspect, and samples resulting in a S/P ratio ≥ 0.40 are considered positive for antibodies to swine influenza virus (H3N2).

Table 3. Presence of antibodies against SIV in different age group in Albania (2009-10)

Age Group	Nr of sera Herd/individual pig	Nr (%) of positive sera (herd/individual pig)			
		H1N1	H3N2		
			Positive	Suspected	Cross reactivity
3-6 months	22	11 (50)	0	4 (18.2)	1(4.5)
	252	45 (17.8)	0	5 (1.9)	1 (0.4)
6-12 months	22	13 (59)	3 (13.6)	0	2 (9.1)
	102	20 (19.6)	3 (2.9)	0	2(1.9)
> 12 months	22	11 (50)	4 (18.2)	0	1(4.5)
(Sows & Boars)	76	14 (18.4)	4 (5.3)	0	1(1.3)
Total	22	13 (59)	4 (18.2)	4 (18.2)	2 (9.1)
	430	79 (18.4)	7 (1.6)	5 (1.2)	4 (0.9)

3. Results and Discussion

The prevalence of seropositivity against SIV during 2007-08 was 19.3% (89/460), seronegativity 80,7% (371/460). After the tests were performed the obtained results of second and third year of investigation were compared with each other in order to know: What SIV subtypes circulate at a specific time at country level? (Subtype H1N1 and H3N2). Are there any differences between the previous examining years in subtype circulations? Seroprevalence of the subtype H1N1 in the third year of investigation was higher than seroprevalence of subtype H3N2. During 2008-09, the prevalence of seropositivity against the swine H1N1 virus was 12.2% (56/459) and the swine H3N2 virus was 8.9% (41/459), and suspected cases were 2,6% (12/459). The cross reactivity was 5.7% (26/459). During 2009-10, the prevalence of seropositivity against the swine H1N1 virus was 18,4% (79/430), the swine H3N2 virus was 1,6% (7/430), and suspected cases were 1,2% (5/430). The cross reactivity was 0.9% (5/430). The data are mirrored in a more detailed way in the table nr.1-3 above. Only six out of twenty two herds (see Table.4 below) were the same herds that we have examined in two successive years of investigation (2008-09 and 2009-10).

The evaluated results obtained after performing the tests demonstrated that two SIV subtypes (H1N1 and H3N2) were circulating in herd level (herd 6). The herd, which is positive for a given SIV subtype in one year may be negative to a given subtype next year

(herd 2, 4 and 5) or may be positive for another SIV subtype (herd 1).

During a three-year investigation period we have tested a total of 1349 samples, 696 of which belonged to an age group of 3-6 months (see table 5 above) with a 11.3% prevalence of seropositivity (or 152 positive sera), 516 samples belonged to an age group of 6-12 months with a 6.8% prevalence of seropositivity (or 92 positive sera), whereas 137 out of 1349 were part of an age group of above 12 month with a 2% prevalence of seropositivity (or 28 positive sera). The findings showed that pigs from the age of 3 to 6 months had the highest prevalence of seropositivity. This effect of the age group on the prevalence of seropositivity can be explained mainly considering the higher possibility of infection during the first months of life due to the lack or absence of maternal antibodies and a naive immune system.

Table 4. Circulation of SIV subtypes in herd level (2008-09 & 2009-10)

Herd/District	Nr of sera/herd	Nr of positive & suspected sera (the same herd 2008-09)			Nr of positive & suspected sera (the same herd 2009-10)		
		H1N1 positive	H3N2 positive	H3N2 suspected	H1N1 positive	H3N2 positive	H3N2 suspected
1) -Levan (Fier)	20	-	-	4	12	-	-
2) -APoci (Gjiroka.)	20	-	2	-	-	-	-
3) -Cerrik (Elbasan)	20	-	-	-	-	-	-
4) -Vau-D (Shkoder)	20	-	1	-	-	-	-
5) -Shelqet (Shkoder)	20	-	-	4	-	-	-
6) -Bushat (Shkoder)	30	16	3	1	-	-	-

Table 5. Swine influenza : Positive sera according to age group

Year	**Age group**	**Nr of sera age group/total**	**Positive sera H1N1+ H3N2**	**% of positive sera Age group / total**
2007-08	3- 6 months	237/460	75	31,6% / 16,3%
	6 -12 months	221/460	14	6,3% / 3%
	> 12 months	2/460	0	0
2008-09	3- 6 months	207/459	32	15,4% / 6,9%
	6 -12 months	193/459	55	28.4% / 11,9%
	> 12 months	59/459	10	16.9%/2.2%
2009-10	3- 6 months	252/430	45	17,8% / 10,5%
	6 -12 months	102/430	23	22,5% / 5,3%
	> 12 months	76/430	18	23,6%/4,2%
Total	3- 6 months	696 / 1349	152	21,8% / 11,3%
	6 -12 months	516 / 1349	92	17,8%/6,8%
	> 12 months	137 / 1349	28	20,4% /2%
	Age group	1349	272	20,1%

Table 6. Swine influenza : Positive sera according to husbandry

Year	**Farming type**	**Nr of sera extensive, intensive/total**	**Positive sera H1N1+ H3N2**	**% of positive sera Ext., ent. / Total**
2007-08	Extensive	180 / 460	14	7,8% / 3%
	Intensive	280 / 460	75	26,8% / 16,3%
2008-09	Extensive	255 / 459	29	11,4% / 6,3%
	Intensive	204 / 459	68	33,3% / 14,8%
2009-10	Extensive	277 / 430	58	20,9% / 13,5%
	Intensive	153 / 430	28	18,3% / 5,5%
Total	Extensive	712/1349	101	14,1% / 7,5%
	Intensive	637/1349	171	26,8% / 12,7%
	Extensive & Intensive	1349	272	20,1%

In Albania we have two farming types of pigs, intensive and extensive, thereby, in our study we have attempt to evaluate the percentage of SIV infection influenced by the husbandry type of pigs (see table 6 above). In total we have tested 712 samples of sera from extensive farms, and 101 (or 7,5%) of them were

positive, whereas 172 (or 12,7%) out of 637 samples from intensive farms were positive. The obtained result in this case has been intriguing, therefore we suggest that the high prevalence of seropositivity in intensive farming is probably due to the higher density of pigs than in extensive farming, moreover, the presence of other viral respiratory infections such as PRRS and PCV2 in intensive farms can reduce immune protection and make pigs more vulnerable to the SIV infection than in extensive farming.

4. Conclusion

The results of this serological study report that:

- We have a circulation of SIV subtypes H1N1 & H3N2 (We do not vaccinate against SIV).
- The prevalence of SIV Abs is about 20% (Three year investigation : 19.3%, 21.1% and 20%).
- The subtype H1N1 is more prevalent than H3N2.
- In different districts we have different SIV subtype circulation .
- We have not been able to identify a clear model of the SIV infection in different farms.
- The circulation of different SIV subtype in the same herd during a two consecutive years may be as the result of pig movement or "new entry".
- The prevalence of seropositivity in intensive farms was higher than extensive farms.
- The age group of pigs may influence the prevalence of seropositivity
- Epidemiological data obtained from this study, permit us to evaluate the potential risk of swine influenza entry into herds, consequently to consider vaccination as a tool for the protection where indicated. Regardless of these results, we should be aware that swine influenza outbreaks in our pig populations are real.

5. References

1. Arora D. J. S, N' Diaye M, Dea S.: **Genomic study of hemagglutinins of swine influenza (H1N1) viruses associated with acute and chronic respiratory diseases in pigs.** *Arch. Virol.* 1997, **142**:401-412.
2. Bachman P A.: Swine influenza, In: Pensaert M B.: **Virus Infections of Porcine**, *Elsevier Science Publisher B. V*., Amsterdam, 1989, 193-207.
3. Gourreau J M, Kaiser C, Valette M, Douglas A R, Labie J, Aymard M.: **Isolation of two H1N2 influenza viruses from swine in France.** *Arch. Virol*. 1994, **135**:365-382.
4. Ito T, Kawaoka Y, Vines A, Ishikawa H, Asai T, Kida H.: **Continued circulation of reassortant H1N2 influenza viruses in pigs in Japan**. *Arch. Virol.* 1998, **143**:1773-1782
5. Karasin A I, Schutten M M, Cooper L A, Smith C B, Subbarao K, Anderson G A, Carman S, Olsen CW.: **Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes.** *Vir. Res*. 2000, **68** (1): 71-85.
6. Kundin W D.: **Hong Kong A-2 influenza virus infection among swine during a human epidemic in Taiwan**. *Nature.* 1970, **228**: 857-867
7. Lange W.: **Pferde influenza, Virologie, Epidemiologie, Klinik, Therapie und Prophylaxe.** Parey Buchverlag, Berlin. 2000, 190.
8. Mirdita L.: **Virusologjia Veterinare,** *Gripi i Derrave (Influenza Suum).,* Tirane. 2002, 189-192
9. Scholtissek C, Hinshaw V, Olsen C W.: **Influenza in Pigs and their Role as the Intermediate Host**, In: Nicholson K G, Webster R G, Hay A J.: *Textbook of Influenza*, Oxford: Blackwell Science, 1998, 137–145.