**Summary**

Chickens (n = 9) and turkey hens (n = 9) were intravenously infected by an avian influenza A virus H6N2 isolate from a wild duck (10^5 ELD50/bird). Cloacal and oropharyngeal samples were collected on day 0 (prior the infection) and on the 3rd, 5th, 7th, 10th, 14th and 21st days after the inoculation from the infected birds as well as from the control birds (6 turkeys and 6 chickens) and analyzed in a first approach by the virus re-isolation method (based on viral replication in chicken embryos and identification by the haemagglutination inhibition test). By this method, the presence of virus in cloacal samples obtained during the first 10 days after inoculation was confirmed in 5/9 turkey hens, whereas virus was detected only in 2 birds using oropharyngeal samples. Furthermore, only one infected chicken was considered as positive: virus was re-isolated from cloacal samples collected on the 3rd and 5th days and from an oropharyngeal sample collected on the 5th day. The samples of the infected turkey hens collected from the 3rd to the 10th days and of the controls collected on the 0, 3rd and the 7th days and those of chickens (infected or not) collected on the 0, 3rd and 5th days were also analyzed with the Directigen™ Flu A test based on detection of viral proteins with specific monoclonal antibodies. With this test, virus was detected in the same inoculated birds (5 turkey hens and one chicken) but the relative sensitivity to the virus re-isolation method remained moderate: 53.3% whatever the nature of samples, 50.0% and 66.7% for cloacal and oropharyngeal samples respectively, the number of positive samples for the 2 methods decreasing when they were collected after the 5th day post-infection. All negative samples for virus re-isolation gave also negative results for viral antigen detection, except one: the relative specificity was 99.5%. The agreement score between the 2 methods was 95.6% (ranging from 92.1% to 98.4% according to the population of birds and to the nature of samples). These results suggest that the Directigen™ Flu A test may be useful in poultry, particularly for confirming the absence of influenza infection.

Keywords: avian influenza virus, turkey hens, chickens, isolation, Directigen™ Flu A test.

**Résumé**

Détection de la grippe aviaire par isolement direct du virus à partir d’échantillons clocaux et oro-pharyngiens issus de dindes et de poulets infectés expérimentalement. Comparaison avec le test Directigen™ Flu A

Neuf poulets et 9 dindes ont été infectés par voie IV par le virus H6N2 de la grippe aviaire isolé sur des canards sauvages (10^5 ELD / animal). Les prélèvements clocaux et oro-pharyngiens ont été effectués à J0 (avant l’infection) et à J5, J7, 10, 14 et 21 après l’inoculation chez les animaux infectés ainsi que chez les oiseaux contrôles (6 poulets et 6 dindes) et analysés dans un premier temps par la méthode d’isolement du virus (possibilité de réplication virale sur des embryons de poulet suivie d’une identification de la souche par un test d’inhibition de l’hémagglutination). Par cette méthode, la présence du virus dans les échantillons clocaux obtenus durant les 10 premiers jours après l’inoculation a été confirmée sur 5 dindes sur 9, alors que les prélèvements oro-pharyngiens issus de seulement 2 de ces dindes ont été positifs. En outre, seulement un poulet infecté expérimentalement a pu être considéré positif : le virus a été effectivement isolé à partir des prélèvements clocaux réalisés à J3 et J5 et à partir du prélèvement oro-pharyngien réalisé à J5. Les prélèvements réalisés chez les dindes infectées les 10 premiers jours et chez les dindes contrôles les 7 premiers jours et ceux effectués chez les poulets infectés ou non à J3 et J5 ont également été analysés par le test Directigen™ Flu A qui permet la détection de protéines virales reconnues spécifiquement par des anticorps monoclonaux. Par ce test, le virus a été détecté chez les mêmes oiseaux expérimentalement infectés (5 dindes et 1 poulet) mais la sensibilité relative par rapport à la méthode d’isolement viral est restée faible : 53.3% quelle que soit la nature du prélèvement, 50.0% et 66.7% respectivement pour les prélèvements clocaux et oro-pharyngiens, le nombre de résultats positifs par les 2 méthodes diminuant lorsque les prélèvements ont été réalisés au-delà du 5ème jour. La détection des protéines virales s’est révélée négative dans tous les échantillons négatifs pour l’isolement viral sauf pour un prélèvement : la spécificité relative a été de 99.3%. L’agrément entre les 2 méthodes a été de 95.2% (allant de 90.7% à 100% selon la population considérée d’oiseaux et la nature des prélèvements). Ces résultats suggèrent que le Directigen™ Flu A pourrait être utile dans les élevages aviaires surtout en vue d’un diagnostic d’exclusion de la grippe aviaire.

Mots-clés : Virus de la grippe aviaire, dindes, poulets, isolement, Directigen™ Flu A.
Introduction

Wild waterfowl are the primary natural reservoir of influenza viruses that can be infecting domestic birds [1]. Chicken and turkey hens are very sensitive birds [2, 4] and are commonly used in experiments in which avian influenza A virus isolates are intravenously administered [11 - 13]. When low pathogenic strains of avian influenza virus (LPAIV) are used, percentage of re-isolates and period of re-isolation markedly differ from species to species [6, 8, 15]. LAUDERT et al. [6] detect 51.4 % positive turkey hens, 45.7 % ducklings and no positive chickens by re-isolation of H13N2 viruses from cloacal samples. TUMPEY et al. [15] obtain more re-isolates with higher viral titres in turkeys with LPAIV strain H7N2 and they suggest that doses 100 – 250 times higher are necessary for chicken infection. Virus re-isolations from the respiratory and the digestive systems vary from 95.2 % [11] and 43 % [15] to 4 % [14]. Re-isolation of virus is usually observed on the 5th – 7th day post infection [8, 14, 15] and in few cases on the 10th – 14th day [3, 7]. However, these authors find higher titres in oropharyngeal samples than in cloacal samples. According to them, this fact indicates that the avian influenza A viruses reproduce more actively in the respiratory rather than in the digestive tract.

Fast and easy-to-use diagnostic tests for detection of influenza A viral antigen are essential for maintaining the survival of the avian population and for developing measures of prevention, control and eradication of the disease, especially after the wide distribution of strain H5N1. The Directigen™ Flu A test produced by Becton Dickinson is an in vitro immunoenzyme-membrane test obtained from monoclonal antibodies against type-specific influenza A viral proteins, which is designed for application on nasopharyngeal aspirates or swabs from probably infected people. This test applied on chicken oropharyngeal and cloacal samples has allowed the virus detection in clinically healthy hens and in clinically affected hens on the 3rd and the 6th days respectively after the intravenous infection [10, 12]. The test sensitivity was higher in the clinically ill birds than in the clinically healthy birds, and these results are in agreement with those obtained by virus re-isolation. CAPUA et al. [3] reported that positive tracheal specimens with Directigen™ Flu A test are obtained on the 7th - 10th days and on the 5th – 7th days post infection in turkeys experimentally infected with virus at the doses of 10^4 ELD50/0.1 ml and 10^6 ELD50/0.1 ml respectively.

The aim of this experiment was to determine the period in which viruses can be isolated from cloacal and oropharyngeal samples and to determine the sensitivity of the Directigen™ Flu A test for indirect virus detection in chickens and turkeys experimentally infected with LPAIV.

Material and Methods

BIRDS AND EXPERIMENTAL PROTOCOL

The LPAIV H6N2 strain used in the present experiment was isolated from a wild duck (Anas platyrynchos) and had a titre of 10^5 ELD50/0.1 ml [16] (ELD50 is the mean embryo lethal doses causing 50% death rate in inoculated chicken embryos).

Fifteen 30-day old white turkey hens of the Beltsville breed and 15 to 30-days old Decalp chickens were used; 9 turkeys and 9 chickens were intravenously infected with 0.1 ml allantoic fluid from chicken embryos (CE) containing cultivated virus, whereas control birds (6 turkeys and 6 chickens) received the same volume of allantoic fluid from intact CE. The birds were kept in two separate 4 x 4 m² rooms, with 1.8 m feeding and watering fronts, with 13 hours daylight, at 20°C temperature and at 70 % humidity. All birds were not vaccinated.

Cloacal and oropharyngeal samples were collected into sterile vials with cotton swabs from all birds on day 0 (prior infection) and on the 3rd, 5th, 7th, 10th, 14th and 21st day post infection. From 210 samples collected, 108 were from the infected birds and 102 from the healthy birds from the control group and those obtained on the day 0.

METHODS FOR VIRUS DETECTION

Virus re-isolation:

Ten percent (w/v) of a culture suspension [MEM with Penicillin G (2 x 106 U/L), Streptomycin (200 mg/L), Polymyxin B (2 x 106 U/L), Gentamicin sulfate (250 ml/L), Nystatin dehydrate (0.5 x 106 U/L) and Sulphamethoxazole (0.2 g/L)] and 0.5 % fetal beef serum (pH to 7.2 – 7.4) were added to the samples. After manual homogenization and centrifugation (at 800 g, for 10 min, at 40C) the obtained supernatant (0.2 ml) was inoculated to three 9-day old CE. After monitoring for 120 hours both dead and alive CE were cooled at 4°C for 2 hours. The presence of haemagglutinating virus in the allantoic fluid was detected using a haemagglutination test which was carried out using 0.05 ml amounts of isotonic saline buffered with phosphate, twofold dilutions of allantoic fluid (1:2 – 1:4096) and 1% chicken red blood cells. Plates were read at room temperature after 30 minutes. The haemagglutination titre is the highest dilution that causes agglutination of the red blood cells.

In a second step, the virus was identified by a test of haemagglutination inhibition (HI) [2]. The virus suspension was incubated with chicken red blood cells (1% concentration) and with hyper-immune chicken serum (final titre 1:256) according to the method of PEARSON and SENNE [9] at room temperature for 30 minutes. The HI titre is the highest dilution of antisera causing complete inhibition of agglutination with 4 or 8 units of virus.

Directigen™ Flu A test

A fast diagnostic test (Becton Dickinson and Sparks, MD 21152, USA) was used following the instructions of the manufacturer. A total of 72 cloacal and oropharyngeal samples collected on the 3rd, 5th, 7th and 10th day post infection from infected turkeys were studied as well as 54 samples from healthy turkeys collected on day 0 and on days 3 and 7 (18
samples from the 9 birds before the viral inoculation and 36 samples from the 6 controls. As far as chickens were concerned, 36 cloacal and oropharyngeal samples from infected birds were collected on the 3rd and 5th days and 42 samples were collected from the healthy chickens on day 0 (18 samples from the 9 birds before viral inoculation) and from the control birds on days 3 and 5 (24 samples).

STATISTICAL ANALYSIS

The comparison of sensibility, specificity and agreement was carried out by the method of COURTNEY et al. [4].

Results

Although birds did not show any clinical signs during the whole experimental period (from the 3rd to the 21st days post infection), the influenza A virus was re-isolated in 5/9 (55.56%) experimentally infected turkeys and only in 1/9 (11.11%) infected chickens. The virus re-isolation was successfully performed only on oropharyngeal and cloacal samples collected between the 3rd and the 10th days post infection in turkeys and between the 3rd and the 5th days post infection in chickens while samples obtained on the 14th and the 21st days have been always negative. Furthermore, the use of cloacal samples for virus re-isolation has allowed its detection in 5 turkeys (3 of them gave positive results on the 3rd and the 5th days and the 2 others on the 7th and the 10th days) and in one chicken on the 3rd and the 5th days. By contrast, virus re-isolation from oropharyngeal samples was less relevant since only 3 samples gave positive results: 2 from 2 different turkeys the 3rd and the 7th days respectively and one from one chicken on the 5th day only (Table I). No virus was re-isolated from not infected birds (turkeys and chickens). Consequently, the specificity of this method (percentage of negative results obtained from the overall samples collected in not infected birds) on cloacal and/or oropharyngeal samples was 100% (Table II) but the sensitivity of the test (percentage of positive results obtained from the overall samples collected in infected birds from the 3rd to the 10th days post infection) remained relatively low. The highest score (27.8%) was obtained in turkeys when cloacal samples were used and decreased to 5.6% in chickens, whereas the sensitivity was depressed to 5.6% and to 2.8% in turkeys and chickens respectively with the use of oropharyngeal samples.

Oropharyngeal samples from a total of 6 different infected birds (5 turkeys and 1 chicken) gave a positive reaction with the Directigen™ Flu A test on the 3rd day (4/6: 3 from turkeys and 1 from the chicken) on the 5th day (1/6) and on the 7th day (1/6) post infection (Table I). Among the corresponding oropharyngeal samples, only 3 positively reacted on the 3rd day (2/3) and on the 7th day (1/3). All positive results with the Directigen™ Flu A test were confirmed by virus re-isolation except for the oropharyngeal sample obtained on the 3rd day from the positive chicken. Moreover, like for the method of virus re-isolation, a negative reaction was observed in all samples stemming from not infected birds (test specificity: 100%). However, the absolute sensitivity of this test was lower (13.9% in turkeys using cloacal samples) (Table III).

As shown in Table IV, the relative sensitivity of the Directigen™ Flu A test to the virus re-isolation (percentage of positive samples with the Directigen™ Flu A test obtained from the overall positive samples for virus re-isolation) was moderate: 53.3% of positive samples from infected turkeys and chickens for virus re-isolation were also positive with the Directigen™ Flu A test. Although the number of positive oropharyngeal samples for the method of reference was small, the relative sensitivity of the Directigen™ Flu A test to virus re-isolation obtained from these samples rose 66.7% whereas only 50% of positive cloacal samples for the method of reference gave also a positive reaction with the Directigen™ Flu A test. Besides, whatever the considered population of infected birds (turkeys, chickens or both) and the nature of the collected samples (cloacal and oropharyngeal) the relative specificity of the Directigen™ Flu A test to the virus re-isolation was very high (99.5%), i.e. all samples negative for virus re-isolation except one were also negative with the Directigen™ Flu A test. Taken together, the agreements (number of identical scores) between the 2 methods were elevated, 96.1% for all birds and ranged from 94.1% to 98.0% when cloacal and oropharyngeal samples were respectively used.

Discussion

After intravenous infection of chickens and turkeys with influenza A virus H6N2 isolate from a wild duck, the virus multiplied both in the respiratory and the digestive systems and then is excreted. The virus pathogenicity requires penetration into host cells; this first step of infection is haemagglutinin-dependent and for the LPAIV, it is also favoured by trypsin-like proteases, leading to specific viral localization in these tissues. Consequently, the presence of the virus has to be investigated by virus re-isolation or by evidencing the viral proteins on cloacal and oropharyngeal samples from the infected birds. Contrary to previous results of SWAYNE and BECK [14] and TUMPEY et al. [15], the frequencies of positive virus re-isolations in cloacal samples (27.8% in turkeys and 5.6% in chickens and 16.7% in the two populations) were higher than in oropharyngeal samples (5.6%, 2.8% and 4.2% in turkeys, in chickens and in the two populations respectively). Virus could probably survive longer in the digestive system than in the respiratory system because it can migrate and replicate in numerous sites such as small intestine and kidneys [11, 13].

In the present study, the detection of the virus by re-isolation or by the Directigen™ Flu A test was positive in 5/9 turkeys experimentally infected, whereas the presence of the H6N2 strain was confirmed by the two methods in only one chicken. Moreover, the virus excretion was evident by virus re-isolation until the 10th day post infection in the turkeys and until the 5th day in the chickens. These results emphasized the higher susceptibility of turkeys than chickens to the influenza A virus as already reported by LANDERT et al. [15]. Nevertheless, despite the administration of the wild duck H6N2 isolate by the intravenous route, the proportion of
birds really infected, as demonstrated by positive virus re-isolation, remained very low in chickens as well as in turkeys. JONES and SWAYNE [5] have reported that the virus survival was markedly decreased when it was inoculated to specie different from the specie of the virus source; for example, viruses stemming from birds other than chickens are not detected in chickens after experimental inoculations. The adaptation of these viral strains to new specie was incomplete probably because of the absence or a very weak penetration into host cells. Consequently, the low degree of infection induced by the H6N2 strain from a wild duck isolate in turkeys and chickens would result from a defect in adaptation of this viral strain to domestic birds, leading to a weak sensitivity of the virus re-isolation method in the present work.

Although Directigen™ Flu A test was first applied to chicken samples 9 years ago [12], its first approval for detecting of viral antigen by OIE has been recent, for 2 years [2]. In the present study, the numbers of positive birds (5 turkeys and 1 chicken) were identical with the two methods, but the frequencies of positive reactions in cloacal samples from experimentally infected chickens and turkeys were lower with this test than with the method of virus re-isolation and were comprised between 5.6% in chickens to 13.9% in turkeys. Nevertheless, the numbers of positive samples obtained in this work were higher than those previously reported by SLEMONS and BUGH [12] in clinically healthy hens experimentally infected with influenza virus.

Besides, viral antigens were successfully detected with the Directigen™ Flu A test in all cloacal samples positive for virus re-isolation on the 3rd day after the experimental infection (early step of the infection), whereas the correlation of the two methods decreased in turkeys and chickens in the following days. It is probably because viral particles are lysed by the host immune system and because the local concentrations of viral antigens have become too low for positively reacting with monoclonal antibodies whereas the virus re-isolation method is independent of the initial quantity of virus and allows the replication of the survivor viruses.

Oropharyngeal samples positive for virus re-isolation, except one obtained from a chicken, gave also positive reaction with the Directigen™ Flu A test. Consequently, the relative sensitivity of this test to virus re-isolation (percentage of positive samples with the two methods) slightly varied according to the nature of samples: 50% and 66.7% for cloacal and oropharyngeal samples respectively. On the other hand, the relative specificity (percentage of negative samples with the two methods) was high (>99%) whatever the population of birds considered and the type of samples. The corresponding agreement scores between the two methods remained high in this study (around 95%).

As a conclusion, despite a lower sensitivity of this test and a relatively high cost compared to the virus re-isolation method, the Directigen™ Flu A test may constitute a rapid and reliable method for screening the avian influenza A infection in poultry and particularly for excluding it.
### References


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**Table 3:** Sensitivity and specificity of the Directigen™ Flu A test applied on cloacal and oropharyngeal samples collected from experimentally infected birds (9 turkeys and 9 chickens that have received 10^6 ELD50 of the H6N2 LPAIV on day 0), between the 3rd and the 10th days post infection from turkeys, the 3rd and the 5th days post infection from chickens and collected from the not infected birds on day 0 (from all birds) and between the 3rd - 7th days from 6 control turkeys and on the 3rd and 5th days from 6 control chickens.

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<th>All birds</th>
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**Table 4:** Relative sensitivity and specificity and agreement of the Directigen™ Flu A test to the virus re-isolation method.

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