An animal-friendly collection of samples from the upper respiratory tract via drinker-swabs for the detection of avian influenza (H5N1) in poultry flocks

Recogida de muestras del tracto respiratorio superior mediante hisopos bebederos para la detección de la gripe aviar (H5N1) en manadas de aves de corral de forma inocua para los animales

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ABSTRACT

To take tracheal swabs from poultry flocks, for detection of avian influenza viruses is time-consuming and involves a high workload for the staff and a lot of stress for the sampled animals. Therefore, an animal-friendly and at the same time reliable method of collected of samples from poultry flocks is needed. A simple method would be a significant improvement in the control of avian influenza, especially in poultry-intensive regions. Swab samples in/on drinkers can easily be taken. When taking water, every animal with a respiratory infection leaves small amounts of its respiratory secretions (mucus) in/on the drinkers. Even before the first clinical signs appear in a flock, the influenza virus is already in small amounts of mucus particle in the drinkers present. By sampling swabs in/on many drinkers in the barn these smallest amounts of respiratory murus from infected birds in the flock can be detected by PCR-technic. In contrast to the faecal samples, the samples from the drinkers contain only a small amount of DNA and RNA from microorganisms

The PCR results from this field study allow the conclusion that swabs from the drinkers in the barn yield very reliable and very important information about the presence of avian influenza viruses in a poultry population. However, it is important to take swab samples from many drinkers in the barn. An Influenza monitoring with swab samples from drinkers is rapid, sensitive, specific, reliable, inexpensive and can be taken easily by individuals, regardless of the age and the number of birds in the flock. Detecting the avian influenza virus in infected flocks with swab sampling in/on drinkers is an animal-friendly process with an improvement in the statistical significance of the infection status of a flock.

Keywords: avian influenza, H5N1, PCR test, animal-friendly swab sampling, swabs in/on drinkers, influenza monitoring, flock diagnostics

RESUMEN

Tomar hisopos traqueales de las manadas de aves de corral para la detección de los virus de la influenza aviar lleva mucho tiempo y supone una gran carga de trabajo para el personal y mucho estrés para los animales de los que se toman las muestras. Por lo tanto, se necesita un método de recogida de muestras de manadas de aves de corral que sea respetuoso con los animales y, al mismo tiempo, fiable. Un método sencillo supondría una mejora significativa en el control de la gripe aviar, especialmente en las regiones con gran densidad de aves de corral. Se pueden tomar fácilmente muestras con hisopos en los bebederos. Al tomar agua, todo animal con infección respiratoria deja pequeñas cantidades de sus secreciones respiratorias (moco) en/sobre los bebederos. Incluso antes de que aparezcan los primeros signos clínicos en un rebaño, el virus de la gripe ya se encuentra en pequeñas cantidades de partículas de moco en los bebederos presentes. Mediante el muestreo de hisopos en/sobre muchos bebederos en el establo, estas cantidades mínimas de murus respiratorio de aves infectadas en el rebaño pueden ser detectadas por PCR-técnica. En contraste con las muestras fecales, las muestras de los bebederos contienen sólo una pequeña cantidad de ADN y ARN de microorganismos
Los resultados de PCR de este estudio de campo permiten concluir que los hisopos de los bebederos del establo proporcionan información muy fiable y muy importante sobre la presencia de virus de la influenza aviar en una población de aves de corral. Sin embargo, es importante tomar muestras de hisopos de muchos bebederos del establo. La vigilancia de la gripe con muestras de hisopos de bebederos es rápida, sensible, específica, fiable, poco costosa y puede ser realizada fácilmente por particulares, independientemente de la edad y el número de aves de la manada. La detección del virus de la influenza aviar en manadas infectadas con muestras de hisopos en/sobre bebederos es un proceso inocuo para los animales que mejora la significación estadística del estado de infección de una manada.

Palabras clave: influenza aviar, H5N1, prueba PCR, muestreo con hisopos inocuo para los animales, hisopos en/sobre los bebederos, vigilancia de la influenza, diagnóstico de parvadas.

1 INTRODUCTION

Health disorders and infectious diseases of turkeys are mostly associated with severe economic losses. Several pathogens are incriminated as possible causes of many disease complexes of turkeys either alone (mono-causal) or in synergy with different other micro-organisms (multi-causal) or accompanied by non-infectious factors. “Non-infectious” means all factors, which influence the bird health and include house structure, climatic conditions (ventilation, temperature, and litter condition), stocking density, feed and water supply, hygienic condition as well as the knowledge and qualification of the stockman. These factors affect each other and can promote or inhibit the health condition of the flock (Hafez, 2017). Highly pathogenic avian influenza virus (HPAIV) is the major pathogen associated with substantial economic losses in poultry production. Zoonotic AIV strains, in addition, have caused multiple cases of human infections, sparking

influenza pandemic concerns (Webster and Govorkova., 2014). Avian influenza is a respiratory infection. Both HPAI *(**H**igh **P**athogen **A**vian **I**nfluenza)* and LPAI *(**L**ow **P**athogen **A**vian **I**nfluenza)* viruses are released into the environment via intestinal and respiratory tracts excretions after infection (Stallknecht et al., 1990). Each time water is ingested and also, respiratory mucus from the pharyngeal-tracheal area also pass in/on the drinker via the beak (Alexander et al., 1980, Connie Leung et al., 2007). From the time of infection with the virus until the time of first symptoms *(incubation period),* the animal is still drinking water (Munoz-Aguavo et al., 2019). The first symptoms of this H5N1 virus are unexplained, asymptomatic deaths in an otherwise inconspicuous herd. If you wipe with a swab along the drinker in the transition area of the water surface, you pick up possible virus material with the biofilm. But the official way to screen or test a poultry flock for the presence of an influenza infection is to take 60 pharyngeal/cloacal swabs from individual birds.

Avian influenza (AI) has been considered as endemic infection in Europe since 2022 (FLI, 2023). Throughout the whole year influenza H5N1 infections occur not only in wild birds but also in farm poultry *(turkeys, layers, broilers, ducks and geese).* In order to early identify infected poultry flocks, rapid and conclusive sampling and diagnostics are necessary, especially in high populated poultry-dense areas. Single-animal diagnostics in diseased or dead birds is simple and reliable, whereas the examination of a larger poultry flock or poultry population for the presence or absence of avian influenza infection is much more difficult. In flock diagnostics, only a statistically validated number of sampled birds provide a reliable statement about the flock.

Anyone, who has ever sampled a larger number of healthy birds know that, due to fixation and/or sampling, damage to/in the animal can occur during collection. The larger and older the animals, the more difficult to sampling and the more serious the possible damage to the animal or the herd. This applies in particular to pharyngeal/cloacal sampling in a turkey flock just before the slaughter date.

When testing for Salmonella in laying hens faecal samples are collected in small-group housing instead of many individual cloacal-swabs, whereas in alternative poultry farming and in chicken and turkey fattening pairs of socks sample are collected. They allow a simple sample collection and reliable statements about the flock. By this simple and safe testing method Salmonella can be found quickly and thus easily controlled. Currently, no data are available for the examination of flocks for avian influenza.

In this series of investigations, which was performed during the H5N1 epidemic from autumn 2021 to spring 2022, the sample collection from the drinker (Fig. 1a, 1b and1c) was tested for its suitability for flock-testing. Here, up to five round drinkers or up to ten nipples/cups for drinker lines were sampled with a swab. All samples were examined either individually and/or in a pool with a maximum of ten swabs using *polymerase chain reaction (PCR)* – (Suarez et al., 2007) in an accredited veterinary laboratory. Except for one sample collected from a layer-hen farm, all samples derived from turkey farms. In all turkey flocks infected with H5N1-infection, clinical signs were not visible to the farmer prior to the appearance of the first death birds in flock.

Also, Abdelwhab, et al., (2011), tested the use of FTA® filter papers for diagnosis of avian influenza virus. Swab samples obtained from chickens infected experimentally with H5N1 virus and spotted directly onto the FTA® cards allowed a sensitive and straightforward diagnosis by RT-qPCR (Hassan et al., 2022). FTA® cards were also suitable for examination of field samples, although AIV RNA was detected with reduced sensitivity in comparison to direct examination of swab fluids. The use of FTA® cards will facilitate safe transport of samples for molecular diagnosis of AIV avoiding the need for an uninterrupted cold storage. In addition, Abdelsalam, et al. (2011) optimized a primer set for polymerase chain reaction (PCR)-based detection of influenza A viruses H5, N1 and M1 genes that was validated with a panel of influenza A virus reference strains representing H5, H7, H9 and H13. Specificity test was carried out by the use of eight type A AIV subtypes (H5N1, H5N2, H5N9, H7N1, H7N3, H7N7, H9N2 and H13N6). Results showed that this protocol is capable of produce two distinguished bands represents the H5 and N1 genes in the duplex format. The specificity test showed that the used primers do not have cross-reactivity with the AIV subtypes other than H5N1. In addition, Sensitivity test revealed that the duplex format has a similar sensitivity limit as the triplex one.

The European commission has announced new rules (EU, 2003) to harmonise the vaccination of animals against epidemic of avian flue observed in EU. The European Food Safety Agency (EFSA, 2002) reports an unprecedented number of outbreaks between October 2021 and September 2022 across 37 European countries, and about 50 million birds were culled.

The rules of vaccination allow for harmonisation of the use of vaccination in aim to prevent or control the spread of the disease. Therefor a country, which used the vaccination in their in their strategy to eliminate avian influenza, has to implement and

monitoring. This will allow safe movement of birds and products from establishments and zones, where vaccination has taken place, according to a commission statement. The new rules, which are in line with international Standards of the world Organization for Animal Health (WOAH, formerly OIE), entered on 12th March 2023.

2 MATERIAL AND METHODS

The animal-friendly sampling using drinker swabs was inspired, first, by the sample collection with so-called chewing ropes that are used for testing swine herds for the Porcine reproductive and respiratory syndrome (PRRS) virus *(do not bring the swab to the animal, but bring the animal to the swab)* and, second, by the search for coronavirus variants in urban waste water during the COVID-19 pandemic *(exploiting the sensitivity and specificity of the PCR assay method).*

Test (1): Drinker Swabs vs. Tracheal Swabs

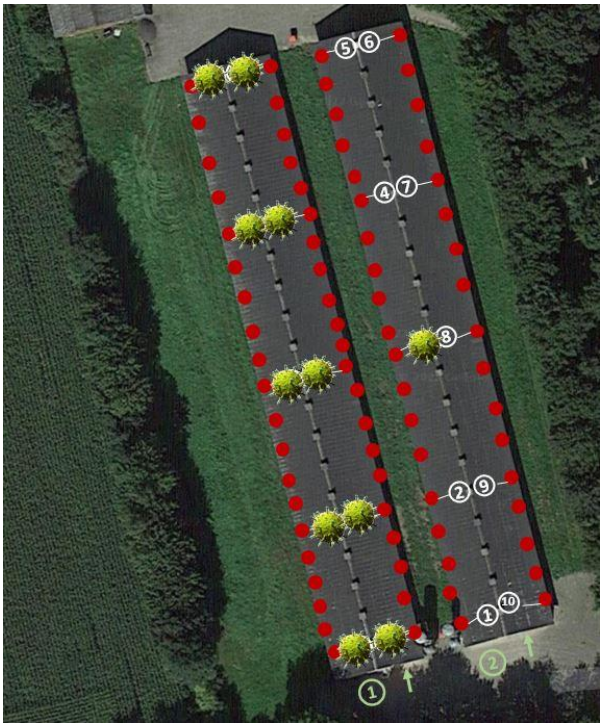
The way of swabbing the drinkers depending to the kind of drinkers in the farm. In the round drinkers (Fig.1a), the swab was wiped with light pressure in the border area to the water or slightly below the water surface at the wall of the drinker over a distance that covers about a quarter of the circumference of the drinker. In the Lubing drinker (Fig. 1b), the swab was wiped approx. fife to ten cm in the border area to the water surface. Five Lubing drinking points were equivalent to one round drinking swab. In the case of nipple drinkers with cups, the swab was first wiped onto the nipple and then into the cup. Ten nipples were equivalent to one round drinking swab. In order to cover the entire herd, it is necessary to distribute the sampling from the drinkers evenly over the barn (Fig. 2).

Are swabs sampled in/on round drinkers same sensitive and reliable than individual pharyngeal swabs from the birds? Therefore, in a flock of turkeys *(5,000 toms, 18th week of age)* with suspected influenza H6N1 infection, one pharyngeal swab was taken from each of five turkeys toms after the appearance of the first respiratory sounds. At the same time, five round drinkers in the flock were each sampled with a swab. The five pharyngeal and the five drinker swab samples were examined with PCR in a pool of five (Table 1).

Figure 1a: Demonstration of taking a swab sample from a Round drinker

Figure 1b: Demonstration of taking a swab sample from a Lubing drinker

Figure 2: Points in the barn of even distribution for swab collection



Test (2): Influenza A-PCR vs. Influenza H-PCR

In the first step of the laboratory diagnostic evaluation for avian influenza, a PCR test for the surface protein A, which is typical for all influenza A viruses, is performed. If the sample is positive, the haemagglutinin type is determined in a subsequent PCR. Whether the ct-values obtained in influenza A-PCR and H-PCR are comparable, swabs are tested in both PCR. The ct-values (cycle-threshold-value) determined in the PCR give an indication of the amount of virus present in the sample. In present field investigations, the influenza samples taken were not always tested with both PCR test kits. Therefore, ten positive H5N1 swabs were examined at the same time (Table 2)

Test (3): Drinker Swabs vs. Sock Swabs

The drinker samples and the sock samples were taken simultaneously in two farms. In the first farm, 2% of the birds in barn no.1 *(4,700 toms at 10th week of age)* had already died after H5N1infection, the water and feed intake had declined sharply. In barn no.2 *(right next to it, also 4,700 toms aged 10 weeks)*, neither dead turkeys nor water and feed decline was observed yet. In the second farm *(also two barns next to each other, 3,200 toms aged 16 weeks)*, clinical H6N1 infection *(severe respiratory sounds as well as decline of feed and water consumption)* had been detected in both barns. On both farms, ten drinker swab samples and two sock pairs were collected from each barn. The sock pairs and the drinker swabs were tested as a pool for influenza A virus in PCR (Table 3).

Test (4): AI Rapid Test vs. PCR Test

Does the testing method have an impact on the PCR result? In one turkey flock *(5.000 toms at 18 weeks of age)* with H5N1 infection, ten drinkers were sampled, in another turkey flock *(4,000 toms at 16 weeks of age)* with H6N1 infection also ten drinkers were sampled, each with a swab. The samples from both farms were analyzed as a pool of 10 using both the PCR technique and the rapid antigen test (Table 4).

Test (5): Swab Clean Drinker vs. Swab Contaminated Drinker

In some poultry houses, drinking hygiene is unfortunately poor. In/on the drinkers can be a lot of dirt or a strong biofilm. In how far this dirt or this biofilm has an influence on the PCR result, two H5N1 positive drinker swabs from different farms, were each mixed with 0.5ml of sterile water in a test tube to have approximately equal virus levels in the mixed swabs. One of each of the two H5N1 positive samples was pooled with nine

swabs obtained from an AI negative farm with good drinker hygiene and the two other H5N1 positive samples was pooled with nine swabs obtained from an AI negative farm with poor drinker hygiene. The results are shown in Table 5.

Test (6): Sensitivity of Nipple Drinkers

The drinking water supply in poultry houses is normal provided either via round drinkers or via nipples with cup drinkers. Does the drinking water technology have an influence on the virus detection in the PCR? In this study all turkey houses unfortunately have round drinkers. In order to test nipples with cups, a laying hen Flock, in which an H6N1 infection was broken out, was examined. The flock size was 5,000 layer-hens in the age of 62 weeks and a water supply based on nipples with cups. The house was divided into five compartments, each containing 1,000 layer-hens. The laying performance, which could not be determined for the individual compartments, was reduced by 30%. Three of the five compartments also had birds that were died from influenza H6N1 *(10-20 dead animals daily).* In each compartment, ten swab samples were taken and sent for examination. Five nipples/cups were sampled with each swab. The five pools of 10 were tested for influenza virus by PCR (Table 6).

Test (7): Sensitivity and Specificity of the Drinker Swab Sample

How sensitive and how specific is a drinker swab in a freshly infected flock? In a turkey farm with two identical turkey barns, approximately 10 meters apart *(5,000 toms aged 19 weeks in each barn),* five toms had been found inexplicably dead in barn no.1 during the morning animal inspections. Water and feed consumption were also slightly reduced. No clinical signs had been noted by the farmer the previous evening. In barn no.2 next to it, there were no abnormalities at that time. Because several H5N1 infections had already occurred in the region, ten drinkers in each barn were immediately sampled with a swab and individually examined for viral RNA in the PCR (Figure 2 and Table 7).

Test (8): Influence of Chlorine Dioxide and Free Chlorine

In many poultry farms, a disinfectant is added to the drinking water to prevent possible microbiological growth in the pipes. Insofar as this water additive has an influence on the PCR result, two approved drinking water disinfectants have been tested. According to the Drinking Water Ordinance on the quality of water intended for human consumption (2001), the addition of 1.2mg chlorine/l and 0.4mg chlorine dioxide/l is

permitted for the disinfection of drinking water. In animal husbandry, both chlorine dioxide (ClO2) and free chlorine (Cl2) are used for continuous disinfection of drinker water to kill germs and prevent biofilm formation. Since there is no possibility to work with live influenza viruses in the field, live viruses of a Newcastle vaccine were used.

Both pathogens of avian influenza and the Newcastle disease belong to the Orthornavirae. Influenza is caused by orthomyxoviruses, the Newcastle disease by paramyxoviruses (Palese et al, 1999). Both viruses are enveloped and have a membrane and have their genetic information stored in an RNA. Therefore, results obtained on Newcastle viruses allow conclusions to be drawn about influenza viruses.

This experiment was not carried out in a poultry house, but under laboratory conditions. In three little containers 1,000 doses of ND live viruses *(AviNew Neo, Hitchner strain, Boehringer Ingelheim)* were added to ten liters of drinking water. In order to exclude the influence of atmospheric oxygen, three liters of each batch were filled into an opaque vessel under exclusion of air and stored at +8 °C. The test was performed with chlorine dioxide at a concentration of 0.75mg/l (0.75ppm), free chlorine at a concentration of 7.4mg/l (7.4ppm) and untreated drinking water (OOWV). A swab sample was taken 1 minute, 1 hour, 8 hours, 24 hours and 48 hours after addition of ND viruses and tested for viral RNA in ND-PCR (Table 8).

Test (9): Persistence of Influenza Viruses in the Drinker

How long is influenza RNA detectable in the drinkers? Long-term detection of HPAI virus is not possible as positive flocks are culled immediately. Therefore, to determine a long-term detection, influenza H6N1 and influenza H9N1 infections were used instead.

In the 2021/2022 epidemic, drinker monitoring for influenza viruses was conducted over a six-month period in a turkey-intensive region. Ten drinker swab samples were taken from all turkey farms on Mondays and Thursdays by the farm manager and examined in a laboratory for the presence of influenza viruses using PCR technology. During the investigation period, H6N1 or H9N2 infections occurred in some poultry farms. Since these strains are not notifiable influenza diseases, infected flocks are not culled. Also, antibiotic treatment has no effect on the time course of influenza. Therefore, these farms are suitable for long-term evaluation (Table 9).

Test (10): Results of Drinker Swabs from three H5N1 Infected Turkey Flocks

During the 2021/2022 epidemic, drinker swab samples were collected from several H5N1 infected turkey flocks to test, if they were suitable for influenza herd diagnostics or not. Three results *(farm A, B, and C)* are presented here. All flocks were culled no later than 48 hours after H5N1 diagnosis.

On farm A, H5N1 infection broke out in 20-week-old turkeys. On the farm, a total of 13,500 toms are kept in three identical open barns. All three barns are located directly next to each other. In the middle barn 2, three toms were found dead for unexplained reasons by the farmer in the evening. The next morning another ten and in barn 3, which stands in wind direction, likewise five dead turkeys were found. In barn 1 were no clinical symptoms at the time of sample collections. Throat/cloak swabs were collected from the dead turkeys and taken to the official testing station. Simultaneously, watering swabs were taken from the drinkers in the three barns. Figure 3 shows a site plan and Table 10 lists the results of the drinker swabs.

On farm B, there was an outbreak of H5N1 infection in 18-week-old turkeys. A total of 9,000 toms are kept in four barns on the farm. All four barns are located directly next to each other. In barn 3, four dead toms were noticed in the morning. During the animal inspection in the late afternoon, there were already another 50 dead turkeys in barn 3 and three dead birds in barn 2. The veterinarian collected throat/cloak swabs from the dead animals and took them to the official inspection station. On the following day, there were already 150 dead toms in barn 3, six dead toms in barn 2 and nine dead toms in barn 1. Drinker swabs were taken by the veterinarian in all four barns. The number of drinkers sampled and the results are shown in Figure 4 and in Table 11. At the time of sampling, no clinical signs were detectable in the turkeys in barn 4 on the downwind side.

On farm C, H5N1 infection broke out in 16-week-old turkeys. A total of 12,000 toms are kept in three barns on the farm. Barn no.1 and barn no.2 are right next to each other, while barn no.3 is located approximately 100 meters up the gable from barn no.2. In barn no.2, three dead turkeys had been noticed in the late afternoon. By next morning, another ten birds had died. In barn no.1 and barn no.3, the toms were inconspicuous. Pharyngeal/cloak swabs were taken from the dead animals and transported to the official test lab. Simultaneously, drinker swab samples were taken from all three barns and tested for H5N1. A site plan is shown in Figure 5, the results of the drinker swab are presented in Table 12.

3 RESULTS AND DISCUSSION

Test 1: Both the pool of five pharyngeal swabs and the pool of five drinker-swabs delivered a positive PCR test result. The ct-values of the trachea swabs had a significantly lower viral load (ct-value 35,8) compare to the swabs in/on the drinkers (ct-value 28,6). This could be due to the fact that the symptoms of the H6N1 infection had already been observed in the flock a few days before the sample was taken. Virus-containing respiratory secretions could increasingly accumulate in/on the drinkers over the past few days. This could explain the significantly higher viral load in the swab pool of the drinkers.

Table 1: **PCR-result**

Turkey-Farm	AI-PCR Pool-test			
	H_5	H_7	H_6	H_9
Trachea Swab 1	neg.	neg.	pos. (35,8)*	neg.
Trachea Swab 2				
Trachea Swab 3				
Trachea Swab 4				
Trachea Swab 5				
Drinker Swab 1	neg.	neg.	pos. (28,6)*	neg.
Drinker Swab 2				
Drinker Swab 3				
Drinker Swab 4				
Drinker Swab 5				

PCR-Test Normec LVL GmbH: Influenza A5-A9

Test 2: The ct-values of the ten drinker swabs were positive in both the Influenza A-PCR and the Influenza H5-PCR. The ct-values determined in the Influenza A-PCR were between 30,6 and 35,3 and those in the Influenza H5-PCR between 28,7 and 34,6. The largest difference between the ten samples was only 10% (swab no.2). That means, no significant difference was found between the two tests.

Table 2: PCR-result: Influenza A vs. influenza H using PCR

Swab no.	Swab 1a - 10a	Swab 1b - 10b
	ct-value A	ct-value H_5
1	33,6	30,8
2	34,5	31,0
3	32,1	29,1
4	30,6	30,8
5	31,7	28,7
6	35,3	34,6
7	34,2	31,9
8	35,1	33,6
9	35,0	31,3
10	35,3	32,5

PCR-Test Normec LVL GmbH: Influenza A Indical, Influenza $H_5H_7H_9$ Congen

Test 3: Virus detection from the respiratory tract via drinkers and from the gastrointestinal tract via sock samples was carried out in four different barns. In three barns Influenza symptoms were recognizable and in one barn there was no indication on an AI-infection. The three pairs of socks from the barn with influenza symptoms delivered a positive PCR test result. But all four barns influenza RNA could be detected the pools of the drinking water samples. Even if, according to the literature, more viruses are excreted via the gastrointestinal tract than via the respiratory tract (Heider et al., 1992). The detection of virus RNA in/on the drinkers of these four barns were more sensitive. Presumably, the large amounts of foreign RNA and foreign DNA in the gastrointestinal germs have a negative effect on the influenza virus detection in routine

Table 3: PCR-results: Drinker swab vs. Sock swabs

Farm	Barn no.	AI-Klinik	AI-Stamm	drinker swabs ct-value A	Sock swabs ct-value A
A	1	++	H6N1	pos. (28,5)	pos. (31,1 + 31,2)
	2	+++	H6N1	pos. (29,1)	pos. (28,0 + 28,0)
B	1	+	H5N1	pos. (30,1)	pos. (32,9 + 31,3)
	2	-	H5N1	pos. (37,1)	neg. + neg.

PCR-Test Normec LVL GmbH: Influenza A Indical, Influenza $H_5H_7H_9$ Congen, Influenza H_6 Anicon

Test 4: In order to be able to rule out an influenza A infection as quickly as possible, a rapid antigen test was used. However, the rapid test was negative in both the H5N1-positve farms as well as in the H6N1-infected farm. The subsequent investigation with the PCR technique delivered a positive result. The small amounts of virus in/on the drinkers, which are reflected in the ct-values of 29,1 to 35,0 are too low to produce a discoloration in the antigen rapid test. Although the PCR technique is slower in terms of time, it is significantly more sensitive and safer. According to these results, the rapid test is only suitable for diagnostics on animals.

Table 4: AI rapid test vs. PCR test from drinkers

sample	AI-positiv	PCR-Test		AI rapid Test
		ct-value A	ct-value H5/6	AIV Ag
swabs from drinkers	H_5N_1	32,1	29,1	neg.
	H_5N_1	35,0	31,3	neg.
	H_6N_1	29,7	n.d.	neg.
	H_6N_1	30,6	30,8*	neg.

Influenza A Indical, Influenza $H_5H_7H_9$ Congen; Antigen-Schnelltest FASTest AIV Ag., n.d.= not done

Test 5: The results of the four H5N1 positive drinker swabs, which were mixed with nine negatives slightly with biofilm contaminated swabs and with nine negatives highly with biofilm contaminated swabs in a pool of ten gives a positive PCR-test. The dilution and contamination with biofilm or dirt have a small, but still a negligible impact on a positive H5N1 swab. Even if the ct-value increased by 25% in a positive swab, the impact is still negligible.

Table 5: PCR-results: Swab from clean drinkers vs. swab from contaminated drinkers

One positive drinker swab with virus	Nine negativ drinker swabs with biofilm	single 1 / pool 1+9			
		1	1 + 9	1	1 + 9
		ct-value A	ct-value A	ct-value H5	ct-value H_5
H_5N_1	+++	35,3	33,9	34,6	33,1
H_5N_1	+++	30,6	32,4	30,8	32,5
H_5N_1	+/-	31,7	29,1	28,7	36,7
H_5N_1	+/-	34,2	27,9	31,9	34,9

PCR-Test Normec LVL GmbH: Influenza A Indical, Influenza H5H7H9 Congen; +/- wenig Biofilm, +++ viel Biofilm

Test 6: The assumption that the poultry tends to leave more respiratory secretions, when drinking water from open round drinkers than when drinking water from a nipple with a cup was not confirmed in this trial. All five pools were positive for influenza A-PCR. These results do not indicate the conclusion, whether virus RNA can be found as early as in open drinkers, because as well as the H6N1 infection had been evident on this farm for a few days.

Table 6: PCR-result: Round drinkers vs. Nipple drinkers
(17.000 Layer-hens, 62 weeks old, H6N1-Infektion *(2021/22)*

barn	Number of animals	Klinik (H6N1)	Laying performance	Number of swabs	Influenza A ct-value
1	3.400	+++	-30 %	6*	30,9
2	3.400	+++		6*	27,1
3	3.400	-		6*	34,7
4	3.400	-		6*	31,5
5	3.400	+++		6*	30,7

PCR-Test Normec LVL GmbH: Influenza A Indical, Influenza $H_5H_7H_9$ Congen: * fife nipples/cups per swab

Test 7: In barn no.1 with the five dead toms at the age of 19 weeks, all ten drinker swabs were positive. In the barn no.2 without clinical signs, virus RNA was only detectable in one drinker. The first cloudy (somnolent) turkey were only discernible in barn no.2 on the following day, about 24 hours later. The ten individual swabs were also examined in a pool of ten. Both the pools of ten from barn no.1 and barn no.2 delivered a positive PCR result. Although there was still no evidence of an H5N1 infection in barn no.2 and virus RNA could only be detected in one drinker, the drinker swabs in the pool of ten delivered a positive PCR test result. This result shows how important it is to sample as many drinkers in the barn as possible in order to detect an infection as early as possible (Fig. 2).

Table 7: PCR-results: sensitivity and specificity of the drinker swab samples

	Drinkers per swab	barn 1 5.000 toms	barn 2 5.000 toms
Death toms		**m / e / m 0 / 0 / 5**	**m / e / m 0 / 0 / 0**
PCR-Test		**ct-Wert A**	**ct-Wert A**
swab 01	**1**	**28,0**	**neg.**
swab 02	**1**	**31,8**	**neg.**
swab 03	**1**	**29,6**	**34,8**
swab 04	**1**	**29,9**	**neg.**
swab 05	**1**	**29,3**	**neg.**
swab 06	**1**	**32,1**	**neg.**
swab 07	**1**	**31,6**	**neg.**
swab 08	**1**	**30,6**	**neg.**
swab 09	**1**	**30,3**	**neg.**
swab 10	**1**	**32,7**	**neg.**
swabs 10er Pool	**10**	**30,1**	**37,1**

PCR-Test Normec LVL GmbH: Influenza A; m = morning; e = evening

Test 8: The addition of disinfectants to sanitize the water pipe is a measure frequently encountered in poultry farms. The results show that within 24 hours there was no significant influence on the ND-PCR result. In a time, window of 48 hours, chlorine dioxide and free chlorine have no significant influence on the RNA detection of paramyxoviruses in the drinkers. An RNA detection of influenza viruses in/on drinkers should give rise to similar results. However, this positive RNA detection does not mean that the ND virus in the three vessels are still a life.

Table 8: PCR-result: influence of chlorine dioxid and free chlorine on the virus

Time after dissolve the live vaccine	Chlorine dioxid 0,75 ppm	Free Chlorine 7,5 ppm (VCP)*	Drinking water untreated OOWV**
1 minute	**ct-value 25,8**	**ct-value 27,2**	**ct-value 27,7**
1 hour	**ct-value 28,3**	**ct-value 28,1**	**ct-value 26,8**
8 hours	**ct-value 31,5**	**ct-value 28,9**	**ct-value 29,3**
24 hours	**ct-value 32,2**	**ct-value 29,5**	**ct-value 31,2**
48 hours	**ct-value 32,8**	**ct-value 31,9**	**ct-value 34,1**

PCR-Test Normec LVL GmbH: New Castle BioChek; *Virbac Clean Pipe; **Oldenburgisch Ostfriesischer Wasserverband

Test 9: Influenza viruses of the strains H6N1 and H9N1 were also spread, in addition to the H5N1 virus during 2021/22 epidemic. For this reason, it was only possible to carry out this long-term sampling. All H5N1 positive flocks are culled immediately, while the H6N1 and H9N1 infected flocks recovered and stay indoors until slaughter. In total, Influenza H6N1 infection occurred in six turkey farms and Influenza H9N1 infection in two turkey farms. The turkeys in the infected flocks were between 10th and 20th weeks of age. The drinker samples were taken on Mondays and Thursdays. By the evaluation, the days between two positive sample results were given a positive correlation. In four farms, the animals were already so old that they could no longer show any negative water intake results by the time they were slaughtered. From these results it can be concluded that the influenza virus can be detected in/on the drinkers for at least a period of 12 days. (Table 9). Because the drinker samples were only taken twice a week, the minimum detection limit can also be one to three days longer. Even this positive PCR results does not mean that the influenza viruses are still infectious over the determined period.

Table 9: Swab-results: persistence of influenza viruses in the drinker from fife H_6 and two H_9 positive flocks. Sampling every Monday and Thursday. The days between positive swabs have been positively related.

Farm	**Days with positive PCR-test in drinker***																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	**H_6**	+	+	+	+	+	+	+	+	**slaughtered**											
B	**H_6**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
C	**H_6**	+	+	+	+	+	+	+	+	+	+	+	**slaughtered**								
D	**H_6**	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
E	**H_6**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
F	**H_9**	+	+	+	+	+	+	+	+	**slaughtered**											
G	**H_9**	+	+	+	+	**slaughtered**															

PCR-Test Normec LVL GmbH: Influenza A Indical, Infuenza $H_5H_7H_9$ Congen; Influenza H_6 Anicon

Test 10: Influenza viruses of the highly pathogenic influenza strain H5N1 were detected by the government investigation in these farms. In the 60 pharyngeal/cloacal swabs, taken from a veterinarian of the government, were the HPAI strain H5N1 detected in the state testing laboratory. All farms were culled within 48 hours after official sampling. The results of the drinker swabs taken parallel are presented below.

Table 10: PCR-results farm A: H_5N_1-Infektion, Toms, 20 weeks *(2020/21)*

	Drinkers per swab	**barn 1: 4.500 toms**	**barn 2: 4.500 toms**	**barn 3: 4.500 toms**
Number of dead birds		**m / e / m 0 / 0 / 0**	**m / e / m 0 / 3 / 10**	**m / e / m 0 / 0 / 5**
PCR-Test		**ct-value A**	**ct-value A**	**ct-value A**
swab 01	**1**	**neg.**	**25,7**	**31,5**
swab 02	**1**	**neg.**	**28,1**	**31,4**
swab 03	**1**	**neg.**	**27,1**	**31,9**
swab 04	**1**	**neg.**	**27,8**	**30,2**
swab 05	**1**	**neg.**	**28,8**	**32,0**
swab 06	**1**	**neg.**	**29,1**	**34,2**
swab 07	**1**	**neg.**	**27,1**	**28,6**
swab 08	**1**	**neg.**	**28,0**	**29,5**
swab 09	**1**	**neg.**	**27,4**	**30,2**
swab 10	**1**	**neg.**	**28,2**	**29,5**
Everage value		**-**	**Ø 27,5**	**Ø 30,5**

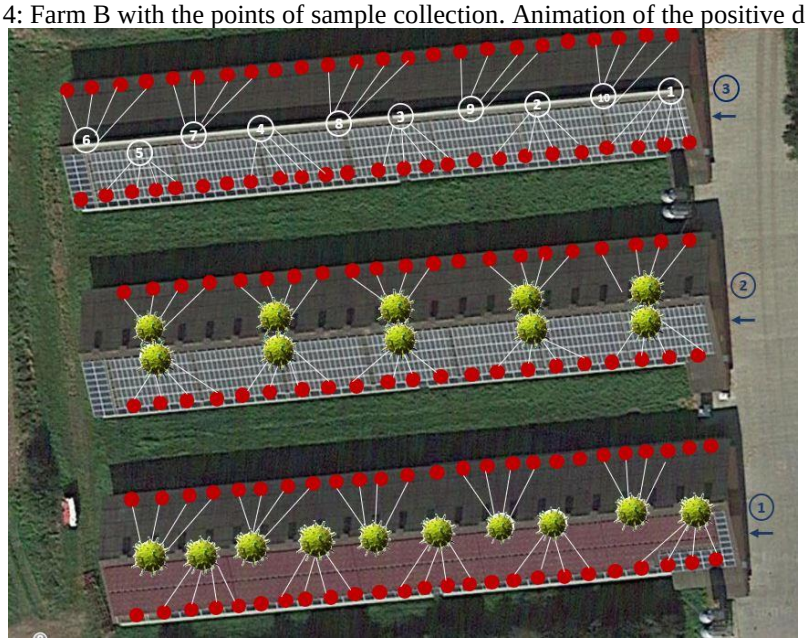
PCR-Test Normec LVL GmbH: Influenza A Indical, Influenza $H_5H_7H_9$ Congen; m = morning; e = evening

Farm A: The male turkeys were infected in the age of 20 weeks. The first entry of the virus took place in the middle barn no.2. Because the barns are littered twice a week with a bedding machine, which started in barn no.3, it is rather unlikely that the virus entry via the litter machine. From barn no.2 the influenza virus was carried downwind to barn no.1. In the barn no.1 five animals had already died from the H5N1 infection on the day the drinker swabs were sampled. In the leeward barn no.3 was no infection detectable. Either no virus RNA could be detected in the drinkers (Fig. 3).

Figure 3: Farm A with the points of sample collection. Animation of the positive drinkers

Farm B: The male turkeys were infected in the age of 18 weeks. Also in this farm, the first entry of the virus did not occur in the barn that was first littered with the bedding machine. From barn no.3 the virus jumped downwind to barn no.2 and then barn no1. The number of sampled drinkers and the results are shown in figure 4 and table 11. In the turkeys in barn no.4, on the leeward side, no symptoms could be identified at the time, when samples were taken. Either no H5N1 virus material could be detected in the drinkers (Fig. 4).

Figure 4: Farm B with the points of sample collection. Animation of the positive drinkers



Farm C: The male turkeys were infected in the age of 16 weeks. In this farm the infection started in barn no.2. Although on farm C the bedding with strow always starts in barn no.1, the infection did not start in this barn. From barn no.2 the H5N1 virus jumped into barn no.1 with the wind direction. Barn no.3 was not infected until culling. The results of the pooled drinker samples from barn no.1 are already positive, although there were still no clinical signs of the infection in the birds (no dead animals, no reduction in feed and water consumption). In barn no.3, all the drinker samples were negative. The results of the drinker swabs correspond very well to the infection process found on the farm (Fig. 5).

Figure 5: Farm C with the points of sample collection. Animation of the positive drinkers

In all the three farms, all drinker swab samples from the barns in which the H5N1 infection started (first infection) were positive. In the barns located on the three farms downwind, the drinker swabs also gave a positive PCR result. In all the three farms, no H5N1 virus material could be identified in the drinker swabs of the barn that were located against the wind direction. These results suggest that influenza viruses are also carried by the wind via particles (dust, feathers, etc.), at least over a short distance (Lüders et al., 2020). Probably just a few days later, the barns facing away from the wind would also have been infected. Due to the fast culling, this did not happen. It should be noted that in all barns in which the first signs of H5N1 infection were already recognizable, H5N1 virus material was detectable in at least one drinker swab. This shows us, that for early identification of an H5N1 virus infection, as many individual drinkers as possible should be covered with a swab.

4 CONCLUSION

Turkeys shed the Influenza viruses in respiratory secretions and contaminated the drinkers early after influenza infection during water intake. Viral RNA is detectable in the drinkers even before the first symptoms appear or animals die from influenza. In this study, viral RNA was detectable in a turkey flock 24 hours before the first clinical signs of H5N1 infection appear. In the PCR-technic the viral RNA was detectable after an AI-infection for at least 12 days in the drinkers. The number and distribution of sampling points in the barn is crucial for the early detection of avian influenza.

To establish herd monitoring based on drinker swabs, however, it is necessary for the method to be scientifically validated. Both the execution of the swabbing of the drinkers and the number of drinkers to be sampled in a herd must be precisely defined. It must be ensured that the drinker swabs have the same statistical certainty as the 60 pharyngeal/cloak swabs.

These field study results from H5N1 infected herds allow the conclusion that drinker swab deliver very reliable and important information on the presence of avian influenza viruses in a poultry population. Swab samples from barn drinkers are rapid, sensitive, specific, inexpensive and an animal-friendly method for monitoring influenza vaccinated poultry flocks.

The new EU-Regulation (EU 2023) of HPAI provides a combination of vaccination and culling. The most important requirement is that a vaccinated herd must be tested once a month with a monitoring for the presence of an infection with an AI field strain. Every vaccinated flock has to be tested once a month until slaughtering. In order to the EU-Regulation it is necessary to have a statistical safe method. In the moment it is the collection of 60 pharyngeal/cloacal swabs per flock. Switching to take swabs in/on drinkers would make influenza monitoring of a vaccinated herd more workable without losing diagnostic quality.

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