Natural and experimental persistence of highly pathogenic H5 influenza viruses in slurry of domestic ducks, with or without lime treatment

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Infections by A/H5 and A/H7 avian influenza viruses (AIVs) can cause acute disease and are therefore notifiable in poultry and wild birds. During winter 2015-2016, several cases of infection caused by highly pathogenic (HP) AIVs belonging to the A/H5N1, A/H5N2 and A/H5N9 subtypes, were detected in south-western France. Throughout winter 2016-2017, several cases of infections mainly caused by A/H5N8 HP AIV (A/Gooses/GD/1/96 clade 2.3.4.4) were detected across Europe. On both occasions, the viruses were widely detected on palmiped farms in France.

This study was designed to evaluate the persistence of A/H5 HP AIV in slurry from various duck productions. This was achieved i) in the laboratory setting by artificially spiking four AIV-free slurry samples with known amounts of A/H5N9 HP AIV and monitoring virus infectivity, with or without lime treatment to achieve pH 10 or pH 12, and ii) by sampling slurry tanks on five naturally A/H5N8 HP-contaminated farms. Experimental results in artificially spiked slurry suggested virus survival for 4 weeks in slurry from Muscovy- or Pekin-duck breeders, and for 2 weeks in slurry from ducks for “foie-gras” production during the assisted feeding period, without lime treatment. Persistence of infectious A/H5N9 HP AIV in all slurry samples after lime treatment at pH 10 or pH 12 was less than 1 week. The A/H5N8 HP AIV persisted in naturally contaminated untreated slurry for 7 weeks. The results obtained provide experimental support for the 60-day storage period without treatment, or the 7-day interval post-lime treatment defined in French regulations for slurry sanitization.
From November 2015 to July 2017, two successive episodes of H5 highly pathogenic avian influenza viruses (HP AIVs) infections occurred on poultry farms in France, mostly in domestic ducks raised for “foie gras” production in south-western France. During the two epizootics, epidemiological investigations were carried out on infected farms and control and biosafety measures were implemented in association with surveillance in order to stop the spread of the viruses. Effluents are known to be an important factor in environmental dissemination of viruses, and suitable effluent management is needed to help prevent the spread of epizootics to other farms, or pathogen persistence at the farm level. The present study was therefore designed to assess how long infectious A/H5 HP AI viruses can persist in naturally or experimentally contaminated fecal slurry samples from ducks, with or without sanitization by lime treatment.

INTRODUCTION

Avian influenza virus (AIV) infections are widespread in many different species of wild and domestic birds. Wild waterfowl (especially in the orders Anseriformes and Charadriiformes) are the natural reservoir of all currently described AIV subtypes, including 16 different hemagglutinin (H1 to H16) and 9 neuraminidase (N1 to N9) subtypes. These birds usually shed the virus through respiratory aerosols or contaminated feces, without developing symptoms (1, 2). These dissemination routes allow transmission to susceptible naive hosts and contamination of natural environments, especially through contaminated feces. Complex natural environments such as lake sediment, mud or sand have the ability to readily adsorb virus particles (3) and may act as a source of influenza viruses in the aquatic habitat (4-6).
Infection of terrestrial poultry, occurring by direct spillover from wild birds or indirect transmission of AIV, is usually asymptomatic or results in mild respiratory disease or a drop in egg production (7). However, more severe symptoms with high mortality have also been associated with several different epizootics of A/H5 and A/H7 highly pathogenic (HP) AIVs. Therefore, A/H5 and A/H7 AIVs are notifiable to OIE, the World Organization for animal health (http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2018/).

From November 2015 to July 2017, two successive episodes of A/H5 HP AIV infections occurred on poultry farms in France, mostly in domestic ducks raised for “foie gras” production in South-Western France. During winter 2015-2016, 81 outbreaks were reported on poultry farms (mostly domestic waterfowl without overt disease) due to one of the three detected subtypes: A/H5N1, A/H5N2 and A/H5N9 HP AIVs, no infection was detected in wild birds. These A/H5 HP AIVs belonged to an H5 genetic lineage significantly different from the A/goose/Guangdong/1/96 lineage, and therefore do not have an assigned clade number (clades have been defined within the A/goose/Guangdong/1/96 lineage only (8, 9)). They presented an unusual HP hemagglutinin cleavage site, a high capacity to spread and induced limited clinical signs in ducks (8, 10). During winter 2016-2017, outbreaks due to the A/H5N8 HP AIV from the A/goose/Guangdong/1/96 lineage (clade 2.3.4.4) among wild birds and poultry were recorded in France and in many countries in Europe (11-14). This A/H5N8 HP AIV induced more obvious clinical signs (nervous symptoms) and higher mortality than the French A/H5 HP AIV detected in 2015-2016. In France, outbreaks affected both poultry (486 cases) and wild birds (55 cases). Domestic ducks raised for “foie gras” production were again the most affected.

Mule duck farming linked with “foie-gras” production is widespread in South-Western France which represents the main production area in the country (15). Briefly, this...
production system entails crossing Pekin female breeder ducks (*Anas platyrhynchos*) with male Muscovy ducks (*Cairina moschata*). After 3-4 weeks rearing in a closed barn and controlled environment, the hybrid ducklings are transferred to the open-range for a 7-to-11-week rearing period, are then moved to closed barns for a 10-to-14-day assisted-feeding period, before finally being transferred to the slaughter house. In most cases, the different rearing steps are performed by specialized farmers, which entails moving the birds from one farm to the next, with specific operational teams and transportation systems involving trucks and crates. The resulting production system therefore combines several aspects: rearing Anseriformes + possible contact with wildlife in the open range + multiple operators and materials + several transfers of animals along the production cycle + need to recycle or spread possibly contaminated slurry. It is essential to manage these factors in order to implement efficient biosafety.

During the two epizootics, epidemiological investigations were carried out on infected farms and control and biosafety measures were implemented in association with surveillance in order to stop the spread of the viruses. Effluents can be an important factor in environmental dissemination of viruses. On most breeding and assisted-feeding farms, feces are collected in the poultry house as slurry (liquid mixture of feces and urine added to litter, feed residues, washing and rainwater). These slurry effluents are different from solid manure, present in most gallinaceous productions, which is a mixture of feces together with substantial quantities of bedding materials, dense enough to be handled as a solid.

Slurry is stored until processing for energy production (methanization) or spreading on pastures as a fertilizer for agronomic use (only approved at certain times of the year). Proper management of slurry is needed to help prevent the spread of epizootics to farms in the vicinity during slurry spreading on fields for soil enrichment, or pathogen persistence at the
Various parameters such as temperature, pH, salinity, humidity, UV light, and physical or chemical composition can affect virus survival in different liquid environments such as saline solution, chicken manure, duck feces, duck meat or lake sediment (4-6, 17-46). A combination of low temperature, neutral pH conditions, and absence of exposure to UV light in a medium enriched with organic material could significantly favor the persistence of infectious AIV particles (4, 6, 17-42, 45, 46). Such conditions prevail in fecal slurry tanks in winter and it is therefore important to define control measures that could be used to sanitize slurry. Slurry sanitization is usually performed by chemical treatment to modify the pH, using lime, sodium hydroxide, formalin or peracetic acid (26, 47). In France, only three methods for slurry treatment are officially accepted in the case of HP AIV detection: treatment in a methanization plant, lime treatment, or storage for at least 60 days before slurry spreading (34). However, only limited information was available from the published scientific literature regarding AIV survival in poultry feces (4, 22, 26, 27, 33, 36, 38, 46), and none pertained to ducks or duck slurry. The present study was therefore designed to assess how long infectious A/H5 HP AI viruses can persist in naturally or experimentally contaminated fecal slurry samples from ducks, with or without sanitization by lime treatment. These data are important to measure the actual efficiency of officially recommended measures for slurry sanitization and help prevent further dissemination of the virus through slurry management during epizootics.

RESULTS

Study on experimentally contaminated slurry

Physico-chemical characteristics

The physico-chemical characteristics of the AIV-free slurries used are presented in Table S1.
The four slurries presented neutral pH, between 6.1 and 7.3.

Experimental spiking of negative slurries with A/H5N9 HP AIV

Prior to spiking with A/H5N9 HP AIV, the four slurries used for the experimental contamination study were confirmed negative for AIV, as no influenza nor contaminating hemagglutinating viruses were detected in embryonated eggs, and no influenza genome was detected by M-rRT-PCR. These slurries were therefore considered suitable for spiking with A/H5N9 HP AIV. A positive control (phosphate-buffered saline PBS) buffer spiked with A/H5N9 HP AIV using the same protocol, then stored in similar conditions, allowed re-isolation of the A/H5N9 HP AI each week until the end of the study (7 weeks post-spiking).

Sequences of A/H5N9 HP AIV is available in the GISAID database (accession number in Table S2).

Persistence of infectious A/H5N9 HP AIV in experimentally contaminated slurry

Figure 1 presents, for each spiked slurry, the results of weekly virus isolation assays and M-rRT-PCRs. All four non-treated spiked slurries allowed A/H5N9 HP AI virus isolation, between 2 and 4 weeks post spiking, although the M gene could be stably detected for the whole duration of the study, up to 7 weeks post spiking. In the four spiked slurries treated with lime milk to achieve pH 10, infectious A/H5N9 HP AIV was isolated on the day of contamination only, for slurries A, C and D (no isolation for slurry B). Discrepantly, detection of the M gene was stable for the whole duration of the study for the four spiked pH 10 slurries tested, and the AIV genome amounts detected were in the same range (2 Ct difference) as those detected in untreated slurries. Following lime-treatment at pH 12, only slurry C allowed the isolation of A/H5N9 HP AI virus, on the day of spiking only, whereas slurries A, B and D did not allow virus re-isolation. The pH 12-condition induced a strong
decrease in M-gene detection, as the M gene was mostly detected on the day of spiking only, and produced undetectable (>40) Ct values afterwards.

Study of naturally contaminated slurry

Persistence of infectious A/H5N8 HP AIV in naturally contaminated slurry

Figure 2 and table 1 present, for each slurry, the results of weekly virus isolation assays or M- and H5-rRT-PCRs. Slurries 2 and 5 did not result in A/H5N8 HP AIV isolation, although the M or H5 genes could be stably detected for at least 3 weeks. A decrease in genome levels was then observed from 3.7 weeks onwards in slurry 2 (H5 detection). Infectious A/H5N8 HP AIV was isolated from slurries 1, 3 and 4, for 1.7 to 7 weeks. Genome detection by M- and H5-rRT-PCR was stable for the whole duration of the study for slurries 1 and 3 (maximum for 10 weeks in slurry 3), and for slurry 4 by M-rRT-PCR (with absence of H5 gene detection in slurry 4 after 5.7 weeks). Infectious viruses other than A/H5N8 HP AIV were also isolated in embryonated eggs. These included an H4 AIV in slurry 2 until 4.7 weeks, Avulavirus 1 (AAvV-1) from slurries 3 and 5 until the end of the study for these samples (10 weeks and 4.6 weeks, respectively) and AAvV-6 from slurry 4 until the end of the study (7.7 weeks). The two detections of AAvV-1 in slurries 3 and 5 were both avirulent, characterized as class II, genotype 1. Isolation in embryonated eggs was reiterated with slurries 2 and 5, after neutralization of the H4 or AAvV-1 contaminants, respectively. These assays did not allow re-isolation of A/H5N8 HP AIV.

For each slurry, sequences encompassing the cleavage site in the H5 gene of each A/H5N8 HP AIV corresponding to the initial isolates were obtained. Regarding the 201 common nucleotides, sequencing data from slurries 1 to 5 were found to be identical, with only a
different nucleotide without changing the correspondent amino acid. All sequences are available in the GISAID or Genbank database (accession numbers in Table S2).

**DISCUSSION**

This study was initiated during the 2015-2016 French H5 AI epizootic, when the A/H5N9 virus was most prevalent (10). As this virus caused only subclinical infection in ducks (8, 10), the initial contamination at the farm level could not be easily dated, and the study was therefore designed based on the experimental contamination of duck slurry that was kept under laboratory conditions at a temperature mimicking the average winter temperature in south-western France. The A/H5N9 and other related A/H5N1 and A/H5N2 AIV (10) were successfully eradicated through massive farm depopulation, mandatory fallowing and increased sanitization during spring and summer 2016. The 2016-2017 epizootic, due to the HP A/H5N8 AI virus, provided the opportunity to complement the experimental A/H5N9 study with the longitudinal follow-up of contaminated farms, as the A/H5N8 virus caused clear clinical signs, allowing us to date the initial introduction of the pathogen at the farm level. The number of naturally infected farms monitored during the course of the second epizootic (n=5) was necessarily limited, due to practical laboratory constraints and the labor intensity of the study protocol, and precludes any statistical evaluation of the duration of virus survival. Furthermore, for biosafety reasons (depopulation, decontamination and disinfection processes), only one initial sample was collected on each farm instead of having weekly samples (repeated sampling in naturally contaminated slurry tanks would furthermore raise the question of how representative and repeatable the sampling process is at farm level).
Persistence of infectious AIV in the environment is influenced by various physical and chemical parameters such as temperature, exposure to UV, pH, presence of chemical agents or detergents, salinity, nature of the matrix and presence of organic material (4-6, 17, 19-25, 27-46). Survival of AIV has also been shown to vary depending on the virus strain (19, 22, 27, 30, 33, 35, 37, 46).

In complex environments with a high content of biological material such as manure or chicken and duck feces, influenza viruses appear to retain infectivity for shorter periods, compare to water (4, 17, 19-22, 24, 27, 28, 32, 35-40, 42, 45, 46, 48). Previous published studies specific to persistence of AIV in manure or poultry feces are presented in Table S3.

In the present study, the persistence of A/H5 HP AIV in naturally contaminated or artificially spiked duck slurries was evaluated at +5°C, with or without lime treatment. The kinetic study was carried out on a small volume of slurry (600 mL), either naturally infected collected from an A/H5HP outbreak or experimentally spiked in the laboratory, stored at +5 °C under biosafety level 3 (BSL3) controlled conditions. Analyses were performed on weekly sub-samples of the stored slurries. This temperature of +5°C was selected as it is representative for the mean winter temperature in south-western France (http://www.meteofrance.com/climat/france/nouvelle-aquitaine/regin09/normales), the largest duck-producing region in the country and which was severely affected by two A/H5 HP AIV epizootics in the winters of 2015-2016 and 2016-2017 (8, 10-14). Additionally, this temperature has previously been described as suitable for virus survival (4-6, 18-21, 23, 24, 28, 32-46, 49) and allowed us to test a “worst case scenario” and obtain a safety margin as survival in actual conditions may be shorter than that observed in the experimental study.

Control of the two A/H5 HP epizootics in France entailed storage of contaminated duck slurry on infected farms and it was therefore important to evaluate experimentally how long...
contaminated slurry would remain infectious so as to define the interval required for slurry 
sanitization. The two pH levels tested in our experimental study, pH 12 and pH 10,
correspond to the limits of the pH range targeted when lime treatment was implemented in
the field (47, 50), as part of the sanitization program designed for infected farms. As the
genome of non-infectious AIV was previously shown to persist in certain environments (48),
two complementary methods of virus detection (viral isolation on eggs and rRT-PCR) were
implemented in the present study. The A/H5N9 HP AI virus used for experimental spiking of
slurries was selected for its high capacity to spread, coupled with limited clinical signs
observed in ducks. It was proven that this virus had spread silently for several months before
it was initially detected (10), and spreading of virus contaminated duck slurry was
hypothesized as one factor possibly contributing to virus dissemination (16). During the
course of the A/H5N9 experimental study, the occurrence of the A/H5N8 HP AI epizootic
provided the opportunity to supplement our experimental study with a survey of duck
slurries naturally contaminated by another H5 HP AIV strain and similarly stored at +5°C.
Our results in naturally contaminated slurries revealed maximum persistence of infectious
A/H5N8 HP AI virus at +5°C for 7 weeks, while the monitoring of experimentally spiked
slurries stored at +5°C demonstrated maximum persistence of infectious A/H5N9 HP AIV for
4 weeks. The A/H5N9 HP AI virus stored similarly, but diluted in PBS buffer, still proved to be
infectious at the end of the experiment (> 8 weeks). Consistently with previous findings
obtained in ducks and chickens (22, 28, 36, 48), the microbial/bacterial flora and the physico-
chemical composition of duck slurry thus seemed to significantly reduce the persistence of
infectious A/H5N9 HP AI virus. The difference in persistence observed in the present study
between the natural and experimental contaminations (7 and 4 weeks of survival,
respectively) could be due to three main hypotheses. First, the difference of persistence
could be due to the composition of slurries collected at different times (winter for naturally
contaminated slurries versus spring for experimentally spiked slurries) that could affect, in
particular the microbiome and physico-chemical properties. To evaluate this hypothesis,
new samples collected during the same period should be tested. Second, the difference in
persistence observed between natural and experimental contaminations could also be due
to the studied A/H5 HP AIV strains. It is of course possible that the A/H5N8 HP AIV strain
belonging to the A/goose/Guangdong/1/96 lineage could be more resistant than the
A/H5N9 HP AIV European strain. Labadie et al. described essential HA amino acids (aa) for
AIV persistence in the environment (51). Here, both the A/H5N8 HP and A/H5N9 HP viruses
exhibited only the lack of insertion of a K residue at position 147 and the presence of a Y
residue at position 543, which are able to induce an increase in the persistence of these
viruses in the environment (51). The other listed aa positions (residues S at position 53, Q at
position 299, A at position 326, (51)) are not present in the viruses included in the present
study. Finally, the last hypothesis is that low residual doses of the A/H5N8 HP AI virus could
more readily infect embryonated eggs than similar amounts of the A/H5N9 HP AI virus,
leading to an apparently longer survival time of the former virus. A similar experimental
study performed with the A/H5N8 HP AI virus would be necessary to support either
hypothesis formally.

In contrast with virus isolation that was only possible for 4 (A/H5N9) to 7 (A/H5N8) weeks,
the AIV genome of both viruses could still be detected for 2 to 5 weeks after extinction of
infectious virus (slurries B and 4, respectively). The Ct values obtained in rRT-PCR for the M
and H5 genes under natural or experimental conditions, demonstrate stable persistence of
the genome in untreated slurries, throughout this period. As a result, whereas AIV genome
detection demonstrates previous presence of AI virus, it cannot be used to assume the
actual presence of infectious AI virus, which must be confirmed with another method such as virus isolation. Our experimental study also investigated two protocols to sanitize duck slurry: lime treatment at pH 10 and at pH 12. Both treatments strikingly reduced virus persistence. Live virus could be isolated only on the day of spiking at pH 10 and at pH 12, in 3 out of 4 and 1 out of 4 assays, respectively. In addition, increasing pH directly increased the degradation of the virus genome in the treated slurry: at pH 10 the genome could be stably detected whereas at pH 12, the genome could not be detected any more as early as one week after lime treatment. These results extend previous knowledge on the effects of basic pH on AIV survival in allantoic fluids, as 15 minutes of treatment at pH 10 or pH 12 was previously shown to have no detrimental effect on AIV hemagglutinating activity (28). Similarly, in another study, an alkaline treatment at pH 11 or pH 13 was virucidal after a 6-hour contact time (32). Interestingly, naturally contaminated slurries presented co-infections with hemagglutinating viruses other than A/HSN8 HP AIV (AAvV-1, AAvV-6 or H4 AIV), that were adventitiously detected upon inoculation of our slurry samples on embryonated eggs. Co-infections are not uncommon and have already been described on duck farms, so making it unsurprising to detect other AIVs or Avulavirus (52). Reassortment was demonstrated to be an important evolutive mechanism during the 2015-2016 epizootics (10), and extensive reassortment could only have occurred when duck farms were co-infected by AIVs belonging to different subtypes. In our study, an H4 AI virus was isolated until 4.7 weeks, and an AAvV-6 virus and two AAvV-1 viruses were still isolated, therefore infectious, at the end of the analyses after 7.7, 4.6 and 10 weeks respectively. These detections are consistent with other published
results demonstrating that the persistence of avian Avulavirus is consistently higher than that of the influenza viruses (4-6, 25).

It is very likely that other viruses with digestive excretion were also present in the studied slurries but went undetected, as they are possibly difficult to isolate using the allantoic route in embryonated eggs. Further studies with different isolation methods would therefore be necessary to more fully assess the role of slurry as a vector contributing to the spread of duck viral diseases.

The physico-chemical composition of slurries could significantly influence the persistence of infectious AIV particles (22, 28, 36, 48). The pH values measured prior to lime treatment in the four slurries used for spiking experiments varied between 6.1 and 7.3, which correspond to the optimal pH range to maintain AIV infectivity (18, 21, 22, 28-34). The chemical composition of the slurries studied here was determined (Table S1). However, differences in composition are difficult to interpret due to the limited reference values regarding duck slurry composition (53, 54). In fact, slurry is a mixture of feces, urine, litter and feed residues, supplemented by washing and rainwater (the latter when slurry tanks are not fully covered): their quantities and compositions are therefore highly dependent on farming practices. In future studies, it could be interesting to analyze the composition of slurries after lime treatment at pH 10 and pH 12, to determine the potential valorization of nitrogen before possibly using treated slurries as fertilizers on agricultural land.

As a conclusion, this study was aimed at evaluating the efficiency of specific sanitation practices implemented in France. Whereas HP AIVs can be disseminated by different routes (respiratory aerosols and aerial transmission, and movement of contaminated equipment and materials), this study was designed to better assess the risk of AIV persistence.
associated with slurry, a common way to store duck feces under farming conditions. To minimize the risk of AIV dissemination through slurries from infected farms, international guidelines have been published (22, 55). In France, three methods are authorized and regulated, namely i) treatment in a methanization structure, ii) treatment with lime so that a pH between 10 and 12 is maintained for seven days, and iii) natural sanitization of slurry by storage for at least 60 days before spreading in the fields (34). Maximum persistence of A/H5 HP AIV for 7 weeks in naturally contaminated slurry without treatment, as demonstrated in our study, provides experimental support for the 60-day interval which is mandatory in France for sanitization by prolonged storage. Similarly, our experiments with spiked and lime-treated slurries showed that no infectious virus was recovered one week post-treatment, which also lends experimental support to the mandatory interval of seven days post lime treatment defined in French regulations. However, the study also presents evidence that other viruses (e.g. AAvV-1) persisted beyond 60 days, suggesting that similar studies could be beneficial if other viruses need to be controlled in the field. Finally, the experimental protocol described here can be used to test the efficiency of new sanitization methods, such as chemical and biological products or physical treatments, and could also be transposed to other pathogens with digestive excretion.

MATERIALS AND METHODS

Selection of duck farms

**AIV-negative farms:** Four AIV-free farms were selected based on their AIV-negative status determined by testing duck flocks with NP-ELISA (Influenza A Ab Test, Idexx) on 20 blood samples and with M-rRT-PCR (described below) on 20 cloacal swabs and 20 tracheal swabs, according to the OIE Manual (56). The farms were selected to represent the diversity of the
different slurry types in duck production in France: one assisted-feeding farm with mule ducks, two with Pekin-duck breeders and one with Muscovy-duck breeders (Table 2). All AIV-negative farms were located outside south-western France and were sampled between March and May 2016.

Naturally infected mule-duck farms: Five contaminated farms for “foie gras” production, during or before the assisted-feeding period, were selected in collaboration with the district animal health authorities (Direction départementale de la protection des populations), based on their positive A/H5N8 HP AIV status, as determined according to the OIE Manual (56). Characteristics of the sampled flocks are summarized in Table 2. All selected farms were located in south-western France and were sampled between January and February 2017.

Collection and storage of fecal slurry samples

All samples were collected with the agreement of both farmers and their official referring veterinarians. All samples were collected before disinfection was implemented in the poultry houses connected to the sampled slurry tanks, in order to avoid possible recent release of disinfectant residues into the sampled slurry.

All samples were collected in accordance with biosafety recommendations to be applied to HP AIV outbreak situations. These measures included wearing a disposable protective suit, gloves and a respiratory mask. Boots and vehicles were disinfected before and after each visit with an approved virucidal disinfectant (Virkon®, LANXESS). All disposable equipment and materials used for sampling were sealed in a plastic bag and destroyed at the farming site by an approved company.
The slurry tanks where samples were collected had not previously been homogenized, in order to keep a possibly higher virus concentration close to the feces adduction duct. No previous sanitizing treatment had been carried out in the tanks in order to keep the titer of possibly infectious virus as high as possible.

For each slurry tank, two 1-liter samples were collected nearest to the adduction duct delivering the fresh droppings into the tank. The sampling location was selected so as to possibly sample the virus last delivered to the slurry tank (i.e. the virus most likely to still retain infectivity at the time of sampling). One liter was taken from the surface of the tank and one liter at a two-meter depth. For surface sampling, a 4-meter long telescopic pole was used, with a 1-liter plastic beaker attached to the end. A bag with a capacity of 1 liter, weighted and equipped with a non-return valve (HydraSleeve GSH 130, EON Products Inc.) was used to collect deep samples.

The surface and deeper slurry samples were transferred to separate screw-capped sterile glass bottles with a capacity of 1.5 liters each. The bottles were sealed and their outside was cleaned with soapy water then disinfected via a spray of Virkon®. The bottles were then packed in double plastic bags, and transferred with paper towel to a water-tight cooler containing frozen ice packs. The samples were directly transferred to an AIV-approved diagnostic laboratory and immediately shipped to the BSL3 containment facility of the French National Reference Laboratory, without any intermediate freezing step.

One slurry sample per farm (AIV-free slurries or naturally contaminated slurries) was sent to the laboratory for storage at +5°C before processing (see below).
AIV-free slurry samples: physico-chemical characterization and validation of AIV-free status

Physico-chemical characterization: the characteristics and composition of slurry samples used for artificial contamination were determined using official methods: pH (EN 12176), electrical conductivity at 20°C and 25 °C (EN 27888), density and dry matter content (%) (EN 13040), organic matter and ash content (%) (EN 13039), organic carbon (%) (ISO 10694), total nitrogen (N %) by the Kjeldahl method (EN 13654), ammoniacal nitrogen (%N/total N) (alkalinization, distillation and titration), organic nitrogen (%N/total N), nitrate nitrogen (%N/total N) (colorimetry), nitrite nitrogen (%N/total N) (colorimetry), calcium (EN 11855), potassium (EN 11855), sodium (EN 11855), phosphorus (EN 11855), sulfur and carbon/nitrogen ratio.

Validation of AIV-free status: the AIV-free status of each slurry used for the artificial contamination study was checked in two steps. First, cloacal swabs were collected from the ducks present in the farms and were tested with M-rRT-PCR. Second, the collected slurry samples were tested again with M-rRT-PCR, and were then analyzed by inoculation on embryonated eggs, to check for a lack of embryo toxicity (irrespective of infectious or chemical origin).

Slurries selected for the study had negative results for all these tests (negative M-rRT-PCR in cloacal swabs and slurry, no embryo toxicity of slurry).

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409  total nitrogen (N %) by the Kjeldahl method (EN 13654), ammoniacal nitrogen (%N/total N)
410  (alkalinization, distillation and titration), organic nitrogen (%N/total N), nitrate nitrogen
411  (%N/total N) (colorimetry), nitrite nitrogen (%N/total N) (colorimetry), calcium (EN 11855),
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419  chemical origin).
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421  cloacal swabs and slurry, no embryo toxicity of slurry).
Artificial spiking with A/H5N9 HP AIV and subsequent lime-treatment

All experiments involving infectious A/H5N9 HP AIV were performed under BSL3 containment conditions. Our BSL3 of the laboratory has been approved by the French National Agency for Medicines and Health Products Safety (ANSM) for the use of specific zoonotic avian influenza viruses (No. ADE-077572017-1).

Artificially spiked slurries: AIV-free slurry samples were artificially contaminated with A/H5N9 HP AIV A/duck/France/150236b/2015 produced as contaminated allantoic fluids derived from specific pathogen-free (SPF) hen eggs as described previously (8, 10). A previous study using slurries naturally infected with A/H5N9 HP AIV had revealed average Ct values of 30.2 ± 3.7 in M-rRT-PCR (57). In order to obtain similar quantities of AIV genome in our experimentally spiked slurries on the first day of the experiment, preliminary tests were performed to determine which virus dose needed to be inoculated: each negative slurry was spiked with several doses of the A/H5N9 HP AIV and then tested with M-rRT-PCR. For each slurry, the dose corresponding to the required quantity of genome was selected. In practice, between 600 µL (for slurries A, C and D) and 6 mL (for slurry B) of A/H5N9 HP AIV were inoculated on 600 mL of slurry, depending on the viral stock used. After spiking with the A/H5N9 HP AIV, the slurry-virus mix was vigorously stirred for a few minutes and was divided into three equal parts. The first part was maintained without any treatment and was subsequently identified as NT (not treated). The other two parts of homogenized contaminated slurry were processed immediately for lime treatment.

Subsequent lime-treatment of artificially contaminated slurry samples: the other two parts of the spiked samples were treated by addition of 45% lime milk (Ca(OH)₂+H₂O; Labat Assainissement), at room temperature for less than 10 minutes, under continuous stirring.
pH was controlled with pH universal indicator strips (Fisherbrand™ pH Indicator 7.0-14 Paper Sticks, Fisher Scientific), so as to achieve pH 10 and pH12pH 12 in the second and third parts, which were identified respectively as T10 and T12 (i.e. for “treated to pH 10” and “treated to pH 12”). Artificially contaminated slurry samples, either untreated or treated with lime, were stored at 5°C ± 3°C for the rest of the study. A positive control consisting in PBS artificially contaminated with A/H5N9 HP AIV, at the same dose as the tested slurries, was prepared and stored similarly. Negative controls consisting of non-contaminated PBS, non-contaminated-slurry without lime treatment, and non-contaminated slurry with lime treatment at pH 10 and pH 12 were prepared. All negative and positive controls were stored as previously described, and collected and tested with the same protocol as the contaminated slurries.

Kinetic study of A/H5 HP survival in duck slurry samples: process for experimentally spiked and naturally contaminated slurries

For naturally contaminated slurry samples, the first day of the kinetic study (D0) was estimated as the last day when fresh fecal material from infected ducks could have been delivered into the slurry tank at the farm level. D0 therefore corresponded to the date when slurry was collected, or to the date when the ducks were depopulated (Table 2). For spiked and lime-treated samples, D0 was the day of artificial contamination and treatment.

All slurry samples, both naturally contaminated and artificially spiked, either lime-treated or not, were sub-sampled weekly (5 mL), after re-homogenization by 5 minutes stirring, and were then analyzed by virus isolation in embryonated eggs, as described below until the virus was no longer isolated for two consecutive weeks. All slurry sub-samples and allantoic
fluids extracted at the end of each isolation assay were also tested using M-rRT-PCR. For the study of naturally contaminated slurry only and in order to confirm the identity of the possibly detected virus, H5-rRT-PCR was also performed from the same slurry sub-samples and allantoic fluids samples.

Detection of AIV genomes by rRT-PCR

RNA extraction: 1 mL each of all collected sub-samples and allantoic fluids was clarified by centrifugation (20,000 x g, 4°C, 10 min). 200 µL of supernatant were collected and viral RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer’s protocol. Briefly, 200 µL of supernatant were mixed with 600 µL of lysis buffer (RLT) containing 6µL of β-mercaptoethanol (Sigma) and left to stand for 10 minutes at room temperature. After addition and mixing with 700 µL of stringent washing buffer (RW1, Qiagen) and 400 µL of 70% ethanol, the liquid was applied to a spin-column in two steps. Washing and elution (50 µL volume) were performed according to the manufacturer’s instructions.

M- and H5-rRT-PCR: the M-rRT-PCR was carried out using a commercial kit (Taq Man one-step rRT-PCR Master Mix, Applied Biosystems) and the Applied Biosystems (ABI) 7500 Fast Real-time PCR system. M-rRT-PCR primers, probe and cycle conditions were previously described by Spackman et al. (57). When samples from naturally contaminated slurries could be detected positive using M-rRT-PCR, due to the presence of other AI viruses, positive samples using M-rRT-PCR from these naturally contaminated slurries were submitted to H5-rRT-PCR. The H5-rRT-PCR was carried out using the same kit and apparatus as for the M-rRT-PCR. H5-rRT-PCR primers, probe and cycle conditions were previously described by Slomka et al. (58).
Analytical protocols used for extraction and rRT-PCR were first validated using this unusual slurry matrix: equivalent results of detection with M-rRT-PCR and H5-rRT-PCR were obtained with both PBS and slurry contaminated with the same dose of H5 virus. All negative controls in PBS were confirmed negative by M-gene real-time RT-PCR (M-rRT-PCR) in all experiments based on naturally contaminated or experimentally spiked slurries. In addition, each sample tested was analyzed with M-rRT-PCR or H5-rRT-PCR using an internal positive control, to confirm the absence of amplification inhibitors. Each real-time RT-PCR run included a dilution series of an appropriate common RNA standard, controlling for the fidelity of the methods and allowing the comparison of results between different runs. The analytical sensitivity of the M-gene rRT-PCR was previously estimated to be 200 and 700 RNA copies per reaction for the A/H5N8 and A/H5N9 viruses, respectively, corresponding to 1000 and 100 Egg Infective Dose 50 (EID50) of the same viruses. Standard deviation of the M-gene rRT-PCR was estimated to be 1.2 Ct (standard values between 31 and 33 Ct), and 1.3 Ct for the H5-gene rRT-PCR (standard values between 34 and 36 Ct) using a standard RNA in series of 20 repeated assays.

**In ovo isolation and characterization of viruses**

**In ovo viral isolation:** Virus isolation was performed according to the official method recommended for AIV (56). After centrifugation (1000 x g, 10-15 min, 2-8°C) of 4 mL of each sub-sample, 1200 µL of supernatant were treated for 15 minutes at 4°C with 1 mL of penicillin, 10 mg/mL of streptomycin, 0.25 g/mL of gentamycin and 0.25 mg/mL of fungizone. Then, 200 µL were inoculated onto the allantoic cavity of 9 to 11-day-old SPF embryonated chicken eggs (ANSES Ploufragan, France). Five eggs were inoculated for each studied sample. Eggs were incubated at 37°C ± 1°C with daily candling. After 5-6 days of
incubation or when embryo mortality was observed, allantoic fluids were collected and their
hemagglutinating activity (HA) was tested. The HA assays were carried out with 1% chicken
red blood cells in PBS, according to the standard protocol (56). A second serial passage on
eggs was carried out similarly on pooled HA-negative allantoic fluids harvested at the end of
the first passage. Virus isolation was deemed negative when no mortality or
hemagglutinating activity in allantoic fluids was detected at the end of the second egg
passage.

For each isolation assay, a negative control test using only inoculation of PBS was
simultaneously checked. All negative controls in PBS were confirmed negative by egg
isolation in all experiments based on naturally contaminated or experimentally spiked
slurries. No embryo toxicity was observed after inoculation of uncontaminated slurry or lime
milk onto control embryonated eggs.

**Confirmation of the identity of the isolated viruses:** samples of fecal material from
conventional poultry may contain a variety of infectious hemagglutinating viruses that could
be re-isolated in embryonated eggs; it was therefore important to confirm the identity of
each virus isolated throughout the kinetic study.

**Virological identification:** hemagglutinating viruses were identified through standardized
hemagglutination inhibition (HI) assays (56), using a panel of anti-avian Avulavirus (AAvV)
and anti-AIV monospecific sera. Antisera used in HI assays were prepared in the authors’
laboratory by immunizing SPF chickens with reference AAvV-1 to 9 except AAvV-5 and H1-
H15 AIV inactivated antigens, obtained either from the AI European Union Reference
Laboratory (APHA Weybridge, UK), or isolated in France (Table S4).
AIV molecular identification by sequencing: the HA2 domain of the HA gene was amplified with a pan-HA RT-PCR (59) or sequences encompassing the cleavage site in the H5 gene were amplified by J3/B2a or Kha1/Kha3 H5-specific RT-PCRs (60). PCR products were sequenced in both directions using the PCR primers and the dye terminator method (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on an automated DNA sequencer ABI 373XL (Applied Biosystems). Sequencing data were assembled and inspected with Mega software. Subtype determination was performed by BLAST analysis on the Influenza virus resource database (61).

Detection of non-AIV contaminating viruses

Molecular identification of non-AIV contaminating viruses: AAvV-1 occasionally detected during the course of the study was pathotyped by sequencing the region encoding the cleavage site of the F protein, using in house RT-PCR with the following primers: NDV F (sense) TAGAAAAAACACGGGTAGAAGA and NDV R (anti-sense) TTGGTWGCRGCAATRCTCTC. Sequencing was performed as described above. The sequence of the cleavage site is correlated with the virulence of AAvV-1 (62, 63).

Neutralization of viral contaminants: during the study of naturally A/H5N8 HP AIV-contaminated slurry, some samples allowed the isolation of viruses different from A/H5 HP AIV. Moreover their in ovo propagation was still associated with significant detection of the H5 genome by H5-rRT-PCR. This situation suggested co-culture of A/H5 HP AIV with another virus, the latter being more easily re-isolated than A/H5 HP AIV. In order to check whether infectious A/H5 viruses were in fact present in such cases, a confirmatory isolation assay was implemented, preceded by viral neutralization of the non-A/H5 contaminating virus. These viral neutralizations were performed by incubating an equivolume mixture of the studied allantoic fluid with a hyperimmune antiserum, monospecific for the virus to be
neutralized for 75 min at 4°C (ANSES-Ploufragan, France). Sera used for neutralization belonged to the same panel of AAvV and AIV monospecific antisera as used in HI (see above). After this neutralization step, virus isolation in embryonated eggs was performed as described above (56).

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Figure 1: Molecular and virological detection of highly pathogenic H5N9 in four experimentally spiked slurries (A to D), from different productions facilities, with or without lime treatment.

Triangles, squares and circles indicate results of M-rRT-PCR from slurry samples without treatment, with lime treatment to achieve pH 10 or with lime treatment to achieve pH 12, respectively (named NT, T10 and T12 in the main text).

Filled or open signs indicate positive or negative viral isolation of H5N9HP, respectively.

The first day of the kinetic study (D0) was the day of artificial contamination and treatment.

Standard deviation of the M-gene rRT-PCR was estimated to 1.2Ct (standard values between 31 and 33Ct) using a standard RNA in series of 20 repeated assays.

Ct: Cycle threshold       Undet: undetermined

Figure 2: Molecular and virological detection of highly pathogenic H5N8 in naturally contaminated slurries.

Diamonds and triangles indicate results of weekly sub-sampled slurries obtained with M- and H5- rRT-PCRs, respectively.

Filled or open signs indicate positive or negative viral isolation of H5N8 HP, respectively.

The first day of the kinetic study (D0) was estimated as the last day when fresh fecal material from infected ducks could have been delivered into the slurry tank, at the farm level.

Standard deviation of the M-gene rRT-PCR was estimated to be 1.2Ct (standard values between 31 and 33Ct), and 1.3 Ct for the H5-gene rRT-PCR (standard values between 34 and 36Ct) using a standard RNA in series of 20 repeated assays.

Ct: Cycle threshold       Undet: undetermined
Table 1: Identification and persistence of isolated viruses other than A/H5N8 HP AIV from naturally infected slurries (no lime treatment).

| Identification of slurry | Egg culture: isolation of other viruses than A/H5N8 HP AIV |
|-------------------------|----------------------------------------------------------|
| Slurry 1                | No isolation of other viruses                            |
| Slurry 2                | Isolation of H4 AIV until 4.7 weeks                      |
| Slurry 3                | Isolation of AAvV-1 until end of study (10 weeks)        |
| Slurry 4                | Isolation of AAvV-1 until end of study (7.7 weeks)       |
| Slurry 5                | Isolation of AAvV-1 until end of study (4.6 weeks)       |
Table 2: Characteristics of AIV-free farms (identified as A to D) and naturally infected farms (identified as 1 to 5).

For artificially contaminated and lime-treated samples, D0 was the day of artificial contamination and treatment.

For naturally contaminated slurry samples, the first day of the kinetic study (D0) was estimated on the last day when fresh fecal material from infected ducks could have been delivered into the slurry tank, at farm level (D0 therefore corresponded to the date when slurry was collected, if ducks were still present in farm when slurry was collected, or to the date when the ducks were depopulated if depopulation was organized before slurry sampling).

| Slurry A | Slurry B | Slurry C | Slurry D | Slurry 1 | Slurry 2 | Slurry 3 | Slurry 4 | Slurry 5 |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Type of production | breeding Muscovy duck | breeding Pekin duck | breeding Pekin duck | 24, Côtes d’Armor | 49, Maine-et-Loire | 49, Maine-et-Loire | 22, Côtes d’Armor | 24, Dordogne | 40, Landes |
| Department | 22, Côtes d’Armor | 24, Dordogne | 49, Maine-et-Loire | 49, Maine-et-Loire | 40, Landes | 40, Landes | 32, Gers | 32, Gers | 65, Hautes-Pyrénées |
| Date of detection | / | / | / | / | 29/12/2016 | 05/01/2017 | 12/12/2016 | 23/12/2016 | 23/01/2017 |
| Date of depopulation | / | / | / | / | 30/12/2016 | 07/01/2017 | 19/12/2016 | 29/12/2016 | 26/01/2017 |
| Collection date | / | / | / | / | 25/01/2017 | 06/01/2017 | 12/01/2017 | 13/01/2017 | 08/02/2017 |
| D0 date | / | / | / | / | 30/12/2016 | 06/01/2017 | 19/12/2016 | 29/12/2016 | 26/01/2017 |
| Beginning of kinetic study in laboratory | / | / | / | / | 1.7 weeks after D0 | 0.7 weeks after D0 | 4.1 weeks after D0 | 2.7 weeks after D0 | 2.6 weeks after D0 |
| Type of slurry tank | concrete | concrete | concrete | concrete | concrete, not closed | geotextile membrane, closed | geotextile membrane, closed | geotextile membrane, closed | geotextile membrane, not closed |
Figure 1: Molecular and virological detection of highly pathogenic H5N9 in four experimentally spiked slurries (A to D), from different productions facilities, with or without lime treatment.
Figure 2: Molecular and virological detection of highly pathogenic H5N8 in naturally contaminated slurries.