

食料・農業・農村政策審議会家畜衛生部会
第35回家きん疾病小委員会

日時：平成22年3月10日（水）13時30分～

場所：第2特別会議室

1 開 会

2 あいさつ

3 議 事

(1) リアルタイムPCR等の新規検査手法を用いた防疫対応への変更

(2) 平成21年愛知県での高病原性鳥インフルエンザの発生に係る疫学調査結果の報告

(3) その他

4 閉 会

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家きん疾病小委員会委員名簿

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(敬称略、五十音順、○：委員長)

I 高病原性鳥インフルエンザに関する特定家畜伝染病防疫指針留意事項の改正について

I-1 家きん卵出荷監視検査におけるリアルタイムPCR法の活用

鳥インフルエンザウイルス検出用リアルタイムPCRキット

1. 品名：FLOCKSCREEN AI-4 Real-time PCR Kit

2. 製造メーカー：x-OVO社（英国）

3. キットの概要

(1) A型に特異的な遺伝子、H5亜型に特異的な遺伝子、H7亜型に特異的な遺伝子、H9亜型に特異的な遺伝子をそれぞれ検出できる。

(2) RNA抽出用としてキアゲンのRNA抽出キットが含まれている。

(3) 特異性は100%

(4) 感度はA型特異的遺伝子をターゲットとした場合、 $10^1 \sim 10^2$ EID₅₀/100uL

H5,H7,H9 特異的遺伝子をターゲットとした場合、 $10^1 \sim 10^{2.74}$ EID₅₀/100uL

(5) 再現性は高い

(6) サンプルは、しょう尿腔液、培養細胞上清、気管スワブ、クローカスワブ、気管・肺・脳等の臓器、糞便

(7) 1キット当たり、最大400反応分

(8) 陽性コントロールとして、βプロピオラクトンで不活化された低病原性鳥インフルエンザウイルスが含まれている。

(9) ワン・ステップ・RT-PCRなので、コンタミのおそれが少ない。



Diagnostic and Control Solutions

The FLOCKSCREEN AI-4 PCR Kit

1. INTRODUCTION

Highly pathogenic avian influenza is one of the most devastating diseases in the animal kingdom. Certain H5 and H7 subtypes can lead to mortalities of up to 100% in infected flocks 48 hours after the onset of clinical signs. The H9 subtype is endemic in many parts of the world and may be associated with mortality and production deficits that compromise commercial poultry farming operations. As avian influenza is spread most commonly by the movement of people and farming equipment from infected to non-infected farms, a rapid and accurate diagnosis of the presence of the infection facilitates the introduction of appropriate control measures that may restrict severely the spread of the virus.

The Istituto Zooprofilattico Sperimentale delle Venezie, (OIE and FAO reference laboratory for avian influenza and Newcastle disease) is a major international centre of expertise for the diagnosis of avian influenza. Experimental protocols developed and validated extensively there for the detection of the major avian influenza subtypes are now available in one convenient kit that may be used as the cornerstone of the molecular diagnostic component of an avian influenza control programme.

The FLOCKSCREEN AI-4 PCR kit contains all the reagents required for the detection of group A, H5, H7 and H9 avian influenza virus RNA from samples of avian origin by real time PCR amplification. According to users' preferences and priorities, after the detection of the presence of group specific RNA (group A), the detection of the three subtypes of major clinical relevance in commercial poultry production systems (H5, H7, H9) can be performed simultaneously, within the same real time PCR run, in separate PCR tubes or wells. The assay is a one step RT-PCR protocol, with the reverse transcription of the target RNA and subsequent PCR amplification occurring within the same reaction mix in the same tube. This reduces significantly sample processing time as well as minimizing the risk of sample cross contamination.

2. INTENDED USE

To be used in a nucleic acid amplification test (real time PCR) to detect, directly, specific virus RNA in specimens of avian origin and viral cultures. The product should form a core part of an overall avian influenza control programme, facilitating the determination of the presence of group A influenza infection in poultry and the rapid follow up of this diagnosis with the possibility to confirm or eliminate the presence of infection with the major AI virus subtypes of clinical and commercial relevance.

Target: primers and probes are directed to a conserved region in the matrix genomic segment (segment 7) and to a conserved region in the subunit HA2 of the haemagglutinin genomic segment (segment 4).
Population to be tested: avian species.



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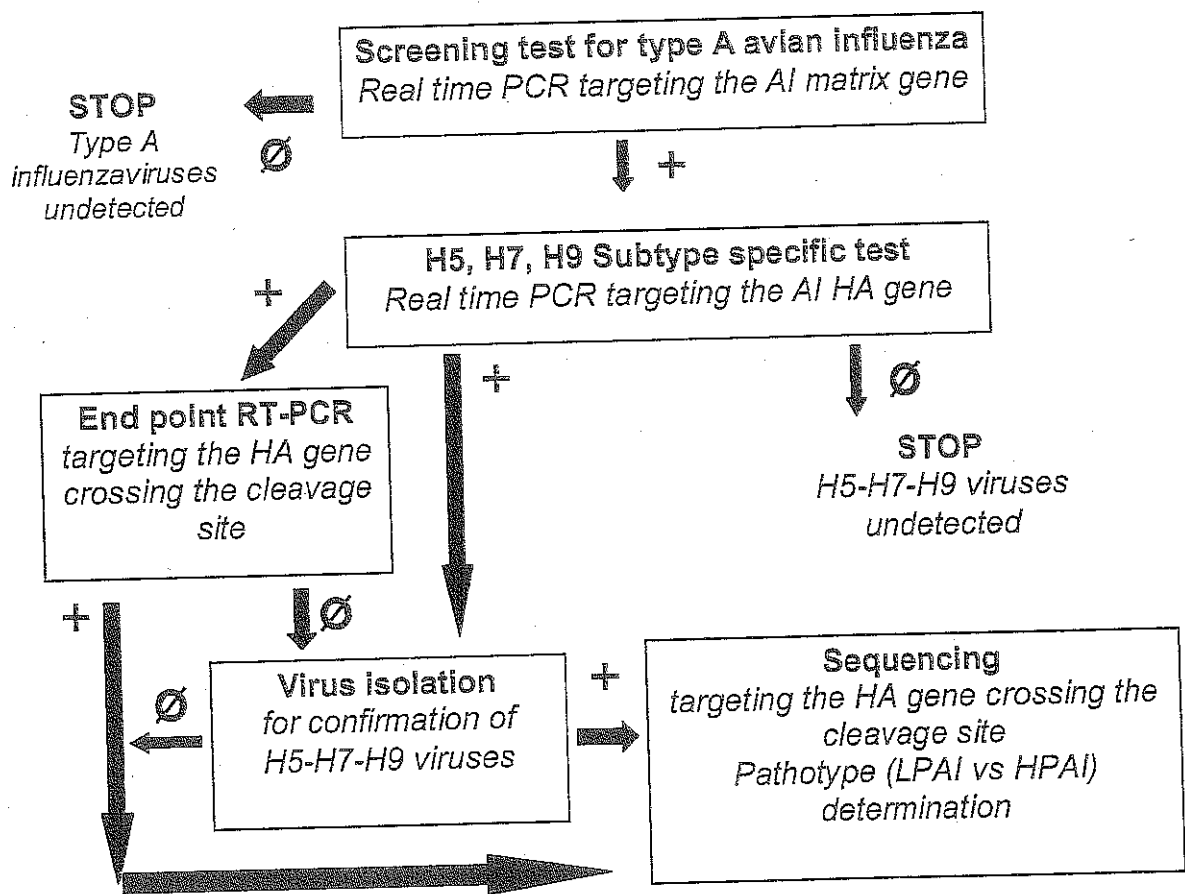
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Specimen type: allantoic fluid of embryonated eggs (viral culture); cell supernatants (viral cultures); tracheal or oro-pharyngeal swab; cloacal swab; internal organs (e.g. trachea, lungs, brain); faecal material.

Important note: the primers and probes were designed on multiple alignments of sequences available in public genetic databases. Due to sequence variability among HA sequences within similar subtypes, primers and probes for H5 and H7 were optimized for the detection of H5 and H7 viruses belonging to the Eurasian/African genetic lineages.

Recommended diagnostic algorithm incorporating the FLOCKSCREEN AI-4 PCR Kit:



Diagnostic algorithm and ancillary/confirmatory tests in case for avian influenza surveillance or confirmation of suspected cases in birds. Legend: AI (avian influenza); HA (haemagglutinin); + (positive test response); Ø (negative test response).



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3. RNA EXTRACTION

This document describes the procedure and the reagents for a real time PCR assay. It does not include reagents for the extraction of the RNA. However, the protocol was evaluated through the application of manual silica column-based RNA extraction technology.

RNA is a fragile molecule. Thus, the quality of RNA obtained from clinical samples may deteriorate rapidly following improper collection, handling and storage. It is strongly recommended that all precautions be taken to avoid RNA degradation and to assure a suitable RNA extraction procedure. Sample collection, handling, storage and RNA extraction laboratory protocols may significantly influence the performances of the real time PCR tests.

Following appropriate validation, x-Ovo Limited recommend the use of the Qiagen RNeasy® MiniKit for optimal RNA extraction (www.qiagen.com, catalogue numbers 74104 and 74106).

4. SPECIFICITY OF THE TEST

The specificity of the primer/probe sets was tested on nucleic acids extracted from a diverse array of microorganisms that may be present naturally in samples of avian origin (Table 1).

The primer and probe set targeting the genomic segment 7 only recognized type A avian influenza viruses. The H5, H7 and H9 primer and probe sets were able to detect RNA of virus strains of their respective subtype only. In this regard, recent avian influenza H5, H7 and H9 virus strains circulating in Europe, Asia and Africa were tested (Table 1).

No positive results were obtained with any of the other organisms listed in table 1.

5. SENSITIVITY OF THE TEST

The sensitivity of the RRT-PCR assay was determined using *in vitro* transcribed RNA and titrated reference viruses.

In terms of HA gene copy number, the Limit of Detection for the H5, H7 and H9 were 10^3 , 10^1 and 10^3 gene copies/ μ l of *in vitro*-transcribed RNA respectively. The sensitivity of the method relative to infectious virus titre detectable ranged between $10^{2.74}$ EID₅₀/100 μ l and 10^1 EID₅₀/100 μ l.

For type A detection, the sensitivity of the method was 10^1 EID₅₀/100 μ l when tested on chicken lungs and faeces and 10^2 EID₅₀/100 μ l when tested on chicken brains.

6. TEST REPEATABILITY

To assess the intra- and inter-assay reproducibility, three different concentrations (high, medium, low) of the reference viruses were tested in triplicate in six different runs performed by two distinct operators on different days. The intra-assay coefficient of variation ranged from 0.12% to 2.64%. The inter-assay coefficient of variation ranged from 2.26% - 4.11%. These values indicate excellent test repeatability.



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7. PCR REACTION EFFICIENCY

To determine PCR efficiency, the Ct values of individual dilutions were plotted against the initial gene copy number to produce typical standard curves. The reaction efficiencies for H5, H7 and H9 genes were 0.97, 0.98 and 0.97 respectively. The correlation coefficient (R2) was greater than 0.99 in all measurements.

Table 1. Microorganisms included in the test specificity assay and the results obtained.

Microorganisms	Strain	Type A	H5	H7	H9
AIV from Europe					
AIV	A/mallard/Italy/05 H5N1	+	+	-	-
AIV	A/swan/Italy/06 H5N1	+	+	-	-
AIV	A/chicken/Italy/98 H5N2	+	+	-	-
AIV	A/duck/Italy/04 H5N3	+	+	-	-
AIV	A/turkey/Italy/80 H5N2	+	+	-	-
AIV	A/chicken/Italy/99 H7N1	+	-	+	-
AIV	A/turkey/Italy/99 H7N1	+	-	+	-
AIV	A/chicken/Netherlands/H7N7	+	-	+	-
AIV	A/turkey/Italy/02 H7N3	+	-	+	-
AIV	A/turkey/Italy/07 H7N3	+	-	+	-
AIV	A/mallard/Italy/94 H7N4	+	-	+	-
AIV	A/turkey/Scotland/70 H9N7	+	-	-	+
AIV	A/Guinea fowl/Italy/07 H9N	+	-	-	+
AIV	A/cockatoo/England/72 H4N8	+	-	-	-
AIV	A/gull/Denmark/02 H16N3	+	-	-	-
AIV from Asia and Russia					
AIV	A/chicken/Vietnam/05 H5N1	+	+	-	-
AIV	A/chicken/Afghanistan/07 H5N1	+	+	-	-
AIV	A/swan/Iran/06 H5N1	+	+	-	-
AIV	A/falcon/Kuwait/07 H5N1	+	+	-	-
AIV	A/chicken/Saudi Ar./07 H5N1	+	+	-	-
AIV	A/turkey/Turkey/05 H5N1	+	+	-	-
AIV	A/avian/Macaw/80 H7N7	+	-	+	-
AIV	A/avian/Pakistan/95 H7N3	+	-	+	-
AIV	A/chicken/Jordan/04 H9N2	+	-	-	+
AIV	A/wild bird/Iran/07 H9N2	+	-	-	+
AIV	A/mallard/Gurjev/82 H14N5	+	-	-	-



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AIV from Africa					
AIV	A/chicken/Egypt/06 H5N1	+	+	-	-
AIV	A/duck/Nigeria/07 H5N1	+	+	-	-
AIV	A/chicken/Ivory Coast/06 H5N1	+	+	-	-
AIV	A/chicken/Benin/07 H5N1	+	+	-	-
AIV	A/chicken/Togo/07 H5N1	+	+	-	-
AIV	A/wild bird/Mali/06 H5N3	+	+	-	-
AIV	A/wild bird/Egypt/07 H7N3	+	-	+	-
AIV	A/wild bird/Egypt/07 H9N2	+	-	-	+
AIV	A/ostrich/SA/01 H10N1	+	-	-	-
AIV	A/wild bird/Mali/06 H11N9	+	-	-	-
AIV from America and Australia					
AIV	A/turkey/Canada/65 H6N2	+	-	-	-
AIV	A/turkey/Ontario/6118/68 H8N4	+	-	-	-
AIV	A/turkey/Wisconsin/66 H9N2	+	-	-	+
AIV	A/duck/Memphis/ H11N9	+	-	-	-
AIV	A/duck/Alberta/60/76 H12N5	+	-	-	-
AIV	A/gull/Maryland/704/77 H13N6	+	-	-	-
AIV	A/shearwater/Aut/79 H15N9	+	-	-	-
Microrganisms AI-unrelated					
APMV-1	Ulster 2C	-	-	-	-
PPMV-1	Pigeon/2875/00	-	-	-	-
APMV-2	Chicken/Yucaipa/56	-	-	-	-
APMV-3	Turkey/1087/82	-	-	-	-
APMV-4	Duck/Hong Kong D3/75	-	-	-	-
APMV-6	Duck/Hong Kong/199/77	-	-	-	-
APMV-7	Dove/United Kingdom/4/75	-	-	-	-
APMV-8	Goose/1053/76	-	-	-	-
APMV-9	Pintail/Italy/493/04	-	-	-	-
IBV	793B serotype	-	-	-	-
IBV	QX-like serotype	-	-	-	-
APV	Type A	-	-	-	-
APV	Type B	-	-	-	-
Bacteria					
<i>Salmonella spp</i>		-	-	-	-
<i>Campylobacter spp</i>		-	-	-	-
<i>Escherichia coli spp</i>		-	-	-	-

Legend: AIV Avian Influenza Virus; IBV Infectious Bronchitis Virus; APV Avian Pneumovirus;

APMV Avian Paramyxovirus; PPMV Pigeon Paramyxovirus.



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8. KIT INSTRUCTIONS

Kit contents

RT-PCR Enzyme pack containing 400 reactions at kit working reaction mixture, 4x1 aliquots of primer/probe mixes for group A, H5, H7, H9 avian influenza sufficient for 400 reactions for each target, 4x1 vials of lyophilised inactivate positive control virus for group A, H5, H7, H9 avian influenza.

Storage conditions

The product should be stored at -20 degrees centigrade to maintain stability and maximise shelf-life. Primers and probes should be reconstituted in 1.2 ml of sterile, RNase free water. Aliquots should be prepared and stored at -20°C protected from light and UV irradiation. Inactivated positive control viruses, once reconstituted in sterile distilled water, should be maintained at +4°C for a few days or at -70°C for longer storage periods. Once reconstituted, stock solutions of positive controls can be used as they are presented or can be diluted 1:10 in sterile PBS (pH 7.4) and stored in aliquots at -70°C. Extracted RNA should be kept at -70°C for long period of time. Alternatively, storage at -20°C can be considered for short periods (e.g. one week). Preparation of small aliquots of positive controls is advisable to avoid frequent freezing and thawing of the sample and the resultant potential degradation.

Safety precautions

The kit does not contain biological hazards. Positive controls contain inactivated (beta propriolactone treatment) low pathogenic avian influenza virus strains. Data relating to all chemicals used are contained in the enclosed product data sheets.

PCR mix preparation

Thaw the reagents. Keep the enzymes refrigerated during the whole process. Carefully mix the reagents and spin for a few seconds. Positive control virus should be resuspended in the volume of distilled water indicated on each respective vial.

Prepare the PCR mix in a sterile, eppendorf-type tube. Use separate tubes for each determination (i.e. one for group A, one for H5, one for H7, one for H9)



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Protocol for the detection of type A influenza viruses

REAGENTS	FINAL CONCENTRATION	$\mu\text{l} \times 1$ reaction
Sterile RNase-free water	/	4.8 μl
Primers/probe mix M 10x	300 nMfor; 300 nMrev; 100nM probe	2,5 μl
2x RT-PCR master mix	1X	12,5 μl
Enzyme mix		0,2 μl
REAGENTS VOLUME		20 μl
RNA		5 μl
FINAL REACTION VOLUME		25 μl

Vortex and spin the mix for few seconds.

Distribute the mix (20 μl) in sterile PCR tubes or in 96 well plates.

Thermal profile for type A influenza virus detection

RT	Initial denaturation	Denaturation	Annealing
50°C	95°C	94°C	60°C
20 min	15 min	45 sec	45 sec
		40 cycles	

Protocol for the detection of H5 AI subtype

REAGENTS	FINAL CONCENTRATION	$\mu\text{l} \times 1$ reaction
Sterile RNase-free water	/	4,8 μl
Primers/probe mix H5 10x	300 nMfor; 300nMrev; 150 nM probe	2,5 μl
2x RT-PCR master mix	1X	12,5 μl
Enzyme mix		0,2 μl
REAGENTS VOLUME		20 μl
RNA		5 μl
FINAL REACTION VOLUME		25 μl

Vortex and spin the mix for few seconds.

Distribute the mix (20 μl) in sterile PCR tubes or in 96 well plates.



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Protocol for the detection of H7 AI subtype

REAGENT	FINAL Concentration	µl x 1 REACTION
Sterile RNase-free water	/	4,8 µl
Primers/probe mix H7-10x	300 nMfor; 900 nMrev; 150 nM probe	2,5 µl
2x RT-PCR master mix	1X	12,5 µl
Enzyme mix		0,2 µl
REAGENTS VOLUME		20 µl
RNA		5 µl
FINAL REACTION VOLUME		25 µl

Vortex and spin the mix for a few seconds.
Distribute the mix (20 µl) in sterile PCR tubes or in 96 well plates.

Protocol for the detection of H9 AI subtype

REAGENT	FINAL CONCENTRATION	µl x 1 REACTION
Sterile RNase-free water	/	4,8 µl
Primers/probe mix H9 10x	300 nMfor; 300nMrev; 150 nM probe	2,5µl
2x RT-PCR master mix	1X	12,5 µl
Enzyme mix		0,2 µl
REAGENTS VOLUME		20 µl
RNA		5 µl
FINAL REACTION VOLUME		25 µl

Thermal profile (identical for H5, H7, H9 determination)

RT	Initial denaturation	Denaturation	Annealing
50°C	95°C	94°C	54°C
20 min	15 min	45 sec	45 sec
		40 cycles	



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Results interpretation§

• Step 1. Real time PCR run validation.

- Check the amplification plot of the positive and negative controls. The positive control should have a clear increase of the fluorescence signal ranging between 9 and 20 Cycle threshold (Ct). The negative control should not have any increase of fluorescence above the threshold.
- In case the controls do not provide the expected results, the run should be considered invalid and the tests repeated.

• Step 2. Interpretation of sample results.

- Samples should be considered as POSITIVE when a clear and regular increase of fluorescence is associated with a Ct ranging from 9 to 37.
- Samples should be considered as NEGATIVE when there is no detection of increasing fluorescence associated with the specific fluorophore (FAM or HEX). Samples revealing a weak increase of fluorescence with an irregular amplification plot and Ct > 37 and < 40 should be considered as non-specific and the sample should be defined as NEGATIVE.
- Samples revealing a regular but weak increase of fluorescence with a Ct > 37 and < 40 should be considered as DUBIOUS/BORDERLINE and the test should be repeated or confirmed by another assay.

§ Derived from validation tests on Applied Biosystems 7300 and Corbett 6000 real time PCR platforms carried out at the Istituto Zooprofilattico Sperimentale delle Venezie, (OIE and FAO reference laboratory for avian influenza and Newcastle disease.



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Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9 Avian Influenza Viruses[†]

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Among the different hemagglutinin (HA) subtypes of avian influenza (AI) viruses, H5, H7, and H9 are of major interest because of the serious consequences for the poultry industry and the increasing frequency of direct transmission of these viruses to humans. The availability of new tools to rapidly detect and subtype the influenza viruses can enable the immediate application of measures to prevent the widespread transmission of the infection. In this study, a novel one-step real-time reverse transcription-PCR (RRT-PCR) was developed to detect simultaneously the H5, H7, and H9 subtypes of AI viruses from clinical samples of avian origin. The sensitivity of the RRT-PCR assay was determined by using in vitro-transcribed RNA and 10-fold serial dilutions of titrated AI viruses. High sensitivity levels were obtained, with limits of detection ranging from 10^1 to 10^3 RNA copies and from 10^1 50% egg infectious dose (EID_{50})/100 μ l to $10^{2.74}$ EID_{50} /100 μ l with titrated viruses. Excellent results were achieved in the intra- and interassay variability tests. The comparison of the results with those obtained from the analysis of 725 avian samples by means of the reference method (virus isolation [VI]) showed a high level of agreement. To date, this is the first real-time PCR protocol available for the simultaneous detection of AI viruses belonging to subtypes H5, H7, and H9, and the results obtained indicate that this method is suitable as a routine laboratory test for the rapid detection and differentiation of the three most-important AI virus subtypes in samples of avian origin.

Influenza viruses are enveloped negative-strand RNA viruses belonging to the family of *Orthomyxoviridae*. Viruses of the *Influenzavirus A* genus cause avian influenza (AI) when infecting birds. AI is a disease of varying severity but may be of great importance for animal health, with serious implications for the poultry industry and, in some cases, for human health. Subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes of influenza A viruses have been identified, and all of these subtypes have been isolated from avian species (10) in most possible combinations. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease in susceptible birds: low-pathogenicity AI (LPAI) and highly pathogenic AI (HPAI). The virulent viruses (HPAI) are restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI.

Only three HA and two NA subtypes (H1, H2, and H3 and N1 and N2, respectively) of influenza A viruses have become established in the human population, although in recent years

AI viruses of subtypes H5, H7, and H9 have caused an increasing number of cases of infection in the human host (6, 16).

AI subtypes H5, H7, and H9 are also of major interest to the poultry industry. The highly pathogenic subtype H5 and H7 viruses have caused several outbreaks with devastating economic consequences (3). Viruses belonging to the H9 subtype are LPAI viruses, but in the last decade, several outbreaks caused by the H9N2 virus have occurred across a wide geographical area, causing serious disease problems in commercial poultry in Iran, Pakistan, and the Middle East (1, 2, 3, 17). In several geographical areas of Eurasia and Africa, these three subtypes have been reported to cocirculate, particularly in areas in which live-bird markets are a common practice and when a superinfection with a distinct subtype occurs in an area in which another subtype is already endemic. This occurrence poses problems for the correct diagnostic interpretation of results in the case of the application of virus isolation (VI) techniques or monovalent PCR assays, in which only the predominant virus may be detected. This can hamper the appropriate management of outbreaks and may result in the application of incomplete intervention strategies.

The significant problems caused for the poultry industry by subtype H5, H7, and H9 viruses and the increased risk of direct transmission of these viruses to humans highlight the need for a highly sensitive, accurate, and rapid test to reveal, as early as possible, the circulation of these viral subtypes in the susceptible avian population.

Conventional AI diagnostic tools (i.e., VI and hemagglutinin

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[†] Published ahead of print on 26 March 2008.

TABLE 1. Viral and bacterial strains used in this study

Pathogen	Strain
Viruses^a	
AIV	A/duck/Italy/1447/05 H1N1
AIV	A/duck/Germany/1215/75 H2N3
AIV	A/psitt/Italy/2073/00 H3N8
AIV	A/cockatoo/England/72 H4N8
AIV	A/mallard/Italy/3401/05 H5N1
AIV	A/chicken/Italy/8/A98 H5N2
AIV	A/duck/Italy/775/04 H5N3
AIV	A/turkey/Italy/80 H5N2
AIV	A/turkey/Canada/65 H6N2
AIV	A/chicken/Italy/1067/V99 H7N1
AIV	A/turkey/Italy/4580/99 H7N1
AIV	A/chicken/Netherlands/03 H7N7
AIV	A/avian/Pakistan/447/95 H7N3
AIV	A/avian/Macaw/626/80 H7N7
AIV	A/turkey/Italy/9289/02 H7N3
AIV	A/mallard/Italy/4818-79/94 H7N4
AIV	A/green winged teal/Egypt/778-14/2007 H7N1
AIV	A/green winged teal/Egypt/778-17/2007 H7N1
AIV	A/chicken/Italy/3981-90/2007 H7N3
AIV	A/turkey/Italy/4527/2007 H7N3
AIV	A/turkey/Ontario/6118/68 H8N4
AIV	A/turkey/Scotland/1/70 H9N7
AIV	A/chicken/Jordan/1436-1529V04/2003 H9N2
AIV	A/chicken/Jordan/1436-554V04/2003 H9N2
AIV	A/turkey/Wisconsin/66 H9N2
AIV	A/ostrich/SA/01 H10N1
AIV	A/duck/Memphis/546/174 H11N9
AIV	A/duck/Alberta/60/76 H12N5
AIV	A/gull/Maryland/704/77 H13N6
AIV	A/mallard/Gurjev/263/82 H14N5
AIV	A/shearwater/Aue/2576/79 H15N9
AIV	A/gull/Denmark/68110/02 H16N3
APMV-1	Ulster 2C
PPMV-1	Pigeon/2875/00
APMV-2	Chicken/Yucaipa/56
APMV-3	Turkey/1087/82
APMV-4	Duck/Hong Kong/D3/75
APMV-6	Duck/Hong Kong/199/77
APMV-7	Dove/United Kingdom/4/75
APMV-8	Goose/1053/76
APMV-9	Fintail/Italy/495/04
IBV	793B serotype
IBV	QX-like serotype
APV	Type A
APV	Type B
Bacteria	
	<i>Salmonella</i> spp.
	<i>Campylobacter</i> spp.
	<i>Escherichia coli</i> strains

^a AIV, AI virus; APMV, avian paramyxovirus; PPMV, pigeon paramyxovirus; IBV, infectious bronchitis virus; APV, avian pneumovirus.

inhibition) are time consuming and require facilities not easily available in some affected areas. Because of their rapidity and sensitivity, molecular tests, such as reverse transcription-PCR (RT-PCR) and real-time RT-PCR (RRT-PCR), are being used more and more by medical and veterinary diagnosticians for the diagnosis of AI (4, 24).

Recently, RRT-PCR assays have been developed for the detection of type A influenza viruses (7, 23) and for the specific diagnosis of H5 and H7 viruses (8, 12, 14, 15, 19, 21, 23, 27). To date, the only published RRT-PCR assay designed for subtype H7 was validated for viruses belonging to the American lineage, and no primer and probe sets are currently available for the identification of subtype H9 viruses.

Here we report the development and validation of a sensitive and specific RRT-PCR assay with hydrolysis-type probes

TABLE 2. RRT-PCR primer and probe sequences

Target	Primer/probe	Sequence (5' to 3') ^a
AI virus subtype H5	H5-For	TTATTCAACAGTGG CGAG
	H5NE-Rev	CCAG(T)AAAGATAGAC CAGC
	H5 probe	CCCTIAGCACTGGCAAT CATG
AI virus subtype H7	H7-For	TTGGITTAGCTTCGGG
	H7-deg Rev	GAAGAA(C)AAGGCC(T) CATTG
	H7 probe	CATCATGTTTCATACTT CTGGCCAT
AI virus subtype H9	H9-For	ATGGGGTTTGCTGCC
	H9-Rev	TTATATACAAATGTTGC AC(T)CTG
	H9 probe	TTCTGGGCCATGTCCA ATGG

^a Parentheses show alternative nucleotides in degenerate primers/probes.

to detect simultaneously subtypes H5, H7, and H9 of the AI virus from clinical samples of avian origin.

MATERIALS AND METHODS

Viruses and bacterial strains. Selected avian viruses and bacteria were used to test the specificity and sensitivity of the RRT-PCR assay (Table 1).

To produce viral working stocks for the standardization of the assay, all avian viruses were propagated in the allantoic cavities of 9- to 11-day-old embryonated fowl eggs, whereas avian pneumovirus type A and B isolates were grown and harvested from tissue cultures. Bacterial strains were cultured and propagated using standard methods (25).

The median egg infectious dose (EID₅₀) of each of the AI viruses used in the sensitivity tests was calculated according to the Reed and Muench formula (16).

RNA extraction. Viral RNA was extracted from clinical samples, supernatant of cell culture, and allantoic fluid by using a Qiagen RNeasy mini kit according to the manufacturer's directions (Qiagen, Hilden, Germany). Two-hundred-microliter samples of allantoic fluid or of phosphate-buffered saline (PBS) suspensions of cloacal and tracheal swabs and samples of feces and organs, as described below, were used in the extraction. RNA was eluted in a final volume of 50 μ l and stored at -80°C.

Primer and probe set design. Viral subtype H5-, H7-, and H9-specific primer and probe sets for conserved regions in the HA2 subunit of the H5, H7, and H9 HA gene sequences were designed (Table 2). Because of the significant sequence variability of the H5, H7, and H9 genes belonging to viruses isolated in different parts of the world, Eurasian and African H5, H7, and H9 influenza viruses were chosen as the main targets for primer and probe design. Multiple alignments of historical and recent H5, H7, and H9 subtypes were performed to minimize primer and probe mismatches. The alignment was performed using, respectively, 166, 81, and 131 HA nucleotide sequences for subtypes H5, H7, and H9. Primers and probes were designed and optimized to have compatible melting temperatures, enabling them to be used with identical thermal profiles. The hydrolysis probes for the H9 and H5 genes contained 6-carboxyfluorescein as a fluorescent reporter dye at the 5' end and 6-carboxytetramethylrhodamine as a quencher dye at the 3' end. The H7 hydrolysis probe was labeled with VIC at the 5' end, and the 3'-end label was 6-carboxytetramethylrhodamine.

RRT-PCR. The reagents contained in a QuantiTect multiplex RT-PCR kit (Qiagen, Hilden, Germany) were used for RRT-PCRs. All but one of the primers targeting the HA gene were applied to the PCR at the optimized concentration of 300 nM each. The exception was the H7-specific reverse primer, which was used at a concentration of 900 nM. Specific fluorescently labeled probes were used at a final concentration of 150 nM. The RRT-PCR took place in a final volume of 25 μ l using a RotorGene 6000 (Corbett, Australia) apparatus. Each PCR tube contained a single primer/probe set (i.e., for H5 or H7 or H9). The identical thermal profile was adopted in order to detect the distinct subtypes simultaneously and within the same run. The following protocol was used for all

TABLE 3. Sensitivity of the method

Strain	Titer of virus stock (EID ₅₀ /100 µl)	Biological matrix	Sensitivity (EID ₅₀ /100 µl)
A/mallard/Italy/3401/05 H5N1 LPAI	10 ^{5.85}	Allantoic fluid	10 ^{1.85}
		Lung	10 ^{1.83}
		Feces	10 ^{1.83}
A/chicken/Yamaguchi/7/04 H5N1 HPAI	10 ^{6.74}	Allantoic fluid	10 ^{1.74}
		Lung	10 ^{2.74}
		Feces	10 ^{2.74}
A/turkey/Italy/4580/99 H7N1 HPAI	10 ^{7.57}	Allantoic fluid	10 ^{1.57}
		Lung	10 ^{1.57}
		Feces	10 ^{1.57}
A/turkey/Wisconsin/66 H9N2 LPAI	10 ⁷	Allantoic fluid	10 ¹
		Lung	10 ¹
		Feces	10 ²

primer/probe sets: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45 s and 54°C for 45 s.

Analytical specificity and sensitivity of the method. The specificity of the primer/probe sets was tested on nucleic acids extracted from a diverse array of microorganisms that may be naturally present in samples of avian origin (Table 1). Each strain used was tested in triplicate.

In the present study, the term "sensitivity of the method" reflects the efficacy of the entire method applied to recover the target organism in the field specimens, including the RNA extraction procedure and the RRT-PCR protocol (26). For this reason, allantoic fluid containing 10-fold serial dilutions of titrated AI viruses belonging to the H5, H7, and H9 subtypes was prepared, and the RNA was extracted and then used for the sensitivity test (Table 3). To establish whether the different types of sample matrices could influence the analytical sensitivity, lungs obtained from specific-pathogen-free chickens were weighed (0.1 g) and homogenized with sterile quartz sand in 1 ml (1:10 wt/vol) of PBS, pH 7.4. Lung homogenates were then blended with 10-fold dilutions of titrated subtype H5, H7, and H9 viruses and processed for RNA extraction. Similarly, feces obtained from specific-pathogen-free chickens were used for sensitivity tests. One-gram samples of feces were weighed and homogenized with sterile quartz sand to obtain a 1:5 wt/vol suspension in PBS. Blending and dilution were performed as described for lung samples.

Evaluation of the analytical sensitivity of the method was done by testing each dilution in five replicates. The sensitivity of the method was determined as the last dilution at which at least 4 of 5 replicates of each dilution was positive.

LoD of the RRT-PCR assay. In the present study, the PCR detection limit reflects the sensitivity of the RRT-PCR procedure, which includes the sensitivity of the primers and probes as well as the preparation of the master mix and the optimization of thermocycling conditions (26). To determine the limit of detection (LoD) of the assay in terms of RNA copy numbers, in vitro-transcribed RNAs of the H5, H7, and H9 genes were analyzed. Briefly, the HA genes of A/chicken/Yamaguchi/7/04 (H5N1), A/turkey/Italy/4580/99 (H7N1), and A/turkey/Wisconsin/66 (H9N2) strains were amplified by RT-PCR and the amplification products were cloned into the PCR-II vector using a dual-promoter TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids with the HA insert were isolated from positive *Escherichia coli* colonies by using a GenElute plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO). The H5, H7, and H9 insert control plasmids were linearized by using the restriction enzyme HindIII (MBI Fermentas, Lithuania) for the H9 and H7 genes and NotI (New England Biolabs, MA) for the H5 gene. The in vitro-transcribed RNA was generated from the T7 promoter by using a RiboMax kit (Promega, Madison, WI) according to the manufacturer's recommendations and then quantified by using a UV BioPhotometer (Eppendorf, Hamburg, Germany).

The number of RNA copies was calculated by following the formula reported in a previous study (11). Tenfold dilutions of the RNA transcripts, ranging from 1 to 10¹⁰ copies/µl, were prepared. The LoD of the assay was determined from three independent replicates.

Intra- and interassay variability. The repeatability of the H5, H7, and H9 RRT-PCR assay was determined using three different concentrations (high, medium, and low) of each viral subtype tested. The selected concentrations were

TABLE 4. Intra- and interassay coefficients of variation

Dilution	Coefficient of variation (%) for subtype:					
	H5		H7		H9	
	Intra-assay	Interassay	Intra-assay	Interassay	Intra-assay	Interassay
High	<1.68	4.06	<1.66	4.02	<1.48	4.11
Medium	<1.58	3.81	<1.35	3.02	<1.74	3.19
Low	<1.16	2.76	<2.64	2.26	<4.11	3.92

10^{5.83}, 10^{4.83}, and 10^{3.83} (H5); 10^{6.57}, 10^{5.57}, and 10^{4.57} (H7); and 10⁶, 10⁴, and 10² (H9) EID₅₀/100 µl. For intra-assay variability, each dilution was analyzed in triplicate. For interassay variability, each dilution was analyzed in six different runs performed by two distinct operators on different days. The coefficient of variation was determined in accordance with previously published guidelines (26).

Detection of virus RNA in samples collected from field-exposed and experimentally infected birds. To evaluate whether or not the RRT-PCR assay could be used as a diagnostic tool in surveillance programs for AI, we analyzed retrospectively by VI and RRT-PCR 725 samples collected from different avian species of poultry ($n = 234$) and wild birds ($n = 491$) during field and laboratory investigations in Eurasia and Africa in 2006 to 2007. The samples consisted of tracheal swabs ($n = 114$), cloacal swabs ($n = 504$), and organs ($n = 107$), consisting of trachea, lungs, intestines, and brain) collected during necropsies. The number of samples analyzed for clinical validation is in accordance with the guidelines proposed in a previous study (5).

The agreement between VI and RRT-PCR results was investigated using Cohen's K statistics (a statistical measure of interrater agreement) and associated evaluation of statistical significance (20). The results were compared with the Cohen's K standard values proposed by Landis and Koch (13).

RESULTS

Specificity, analytical sensitivity, and intra-/interassay variability. The H5, H7, and H9 primer and probe sets were able to detect RNA of virus strains of their respective subtype only. No positive results were obtained with any of the other organisms listed in Table 1.

The sensitivity of the RRT-PCR assay was determined using in vitro-transcribed RNA and titrated reference viruses. In terms of HA gene copy number, the LoDs for the H5, H7, and H9 subtypes were 10⁵, 10¹, and 10³ gene copies/µl of in vitro-transcribed RNA, respectively. To determine the linearity of the reaction and PCR efficiency, the threshold cycle values of individual dilutions were plotted against the initial gene copy number, leading to typical standard curves. The linear ranges of the RRT-PCR assay span within 10¹⁰ and 10² copies/µl for the H7 gene and within 10¹⁰ and 10⁴ copies/µl for the H5 and H9 genes. The reaction efficiencies for H5, H7, and H9 genes were 0.97, 0.98, and 0.97, respectively. The correlation coefficient (R^2) was higher than 0.99 in all measurements.

The sensitivity of the method relative to the detectable infectious virus titer ranged between 10^{2.74} EID₅₀/100 µl and 10¹ EID₅₀/100 µl. The results obtained for the sensitivity of the method in different samples are summarized in Table 3.

To assess the intra- and interassay reproducibility, three different concentrations (high, medium, and low) of each reference virus were tested in triplicate in six different runs performed by two distinct operators on different days. The coefficients of variation within runs (intra-assay variability) ranged from 0.12% to 2.64%. The interassay variability was in the range of 2.26% to 4.11% (Table 4).

TABLE 5. Results of RRT-PCR and VI from clinical samples

RRT-PCR result	VIHI result ^a	No. of samples of subtype:		
		H5	H7	H9
+	+	98	52	24
+	-	43	6	6
-	-	584	667	693
-	+	0	0	2

^a HI, hemagglutinin inhibition.

Detection of viral RNA in samples collected from field-exposed and experimentally infected birds. A total of 725 samples was analyzed for subtypes H5, H7, and H9 by VI and RRT-PCR (Table 5). Of these, 141 samples tested positive for subtype H5 by means of RRT-PCR (4/114 tracheal swabs, 54/504 cloacal swabs, and 83/107 organ samples). For subtype H7, the RRT-PCR assay identified 58 positive samples (44/114 tracheal swabs and 14/504 cloacal swabs), and 30 specimens resulted in positive results for subtype H9 (21/114 tracheal swabs and 9/504 cloacal swabs). The comparison of the results of the two methods, summarized in Table 5, showed agreements of 94.06%, 99.17%, and 98.89% for the H5, H7, and H9 subtypes, respectively. The Cohen's *K* coefficients were 0.80, 0.94, and 0.85, respectively. The difference between the two methods was not statistically significant ($P < 0.01$). The percentage of agreement between the results of RRT-PCR and VI was influenced by the higher number of samples that tested positive by RRT-PCR but negative by VI. However, 43/55 RRT-PCR-positive/VI-negative samples were sequenced and their identities confirmed (data not shown). Only 2 of 26 samples that were positive by VI for subtype H9 tested negative in the molecular assay.

DISCUSSION

Several diagnostic methodologies are currently available for the detection of AI infection, with VI in eggs universally recognized as the gold standard. However, this method is time consuming and requires facilities (e.g., BSL3 laboratories) that are not available in many affected areas. Recently, molecular diagnostic tests have proven themselves to be invaluable as a first step in the identification and control of disease outbreaks. Conventional RT-PCR and RRT-PCR have been applied successfully to the diagnosis of AI (7, 8, 12, 14, 15, 19, 21, 23, 27). In this study, we present data on the development and validation of a real-time hydrolysis probe-based RT-PCR assay for the simultaneous detection of AI viruses belonging to subtypes H5, H7, and H9. Our results prove that the assay is highly specific and sensitive. In a previous study (23), an RRT-PCR assay was developed for the sequences of North American AI virus H5 and H7 subtypes. In that assay, the sensitivity data obtained were comparable to the results described here. However, the protocol described previously was a one-step RT-PCR with different thermal profiles for H5 and H7 detection. In addition to its sensitivity and specificity, the method described in the present paper offers several advantages over conventional diagnostic methods, including rapidity, flexibility, and ease of use. This assay makes results for the three major AI viruses currently prevalent in poultry in large areas of the

world available in approximately 3 h, and the use of a single-step RRT-PCR procedure provides some protection against contamination events. Based on its technical characteristics, this assay could be used for large-scale screening and subtyping of viral RNA during influenza A virus outbreaks and for surveillance programs.

This RRT-PCR assay was developed and validated using the same annealing temperature in order to identify the H5, H7, and H9 subtypes in a single analytical session. In the literature, the use of multiplexed PCRs has been reported as resulting in a decreased sensitivity of the method (22, 28), and the optimization of the concentration of the multiplex PCR components to achieve optimal amplification can pose several difficulties (9). For these reasons, the development of a multiplex assay was not attempted, as it was so important to identify the three subtypes and maximize the assay's performance. The application of this RRT-PCR format was also due to the necessity of having a cost-effective and flexible diagnostic tool that could be easily switched to a single-subtype identification method that would be applicable during the investigation of outbreaks caused by only one of these subtypes. It should also be considered that not all of the existing real-time PCR platforms are capable of detecting more than two fluorophores simultaneously, making a triplex PCR protocol inapplicable.

The suitability of the RRT-PCR test described in the present study as a diagnostic tool to rapidly recognize the three most important HA subtypes of the AI virus is confirmed by the results obtained using samples from birds infected naturally. These clinical samples were obtained from a wide range of avian species and geographical areas during field and laboratory investigations. The assay has been used for monitoring the AI virus in poultry and wild birds, and it has proved capable of identifying the presence of several distinct genetic lineages of subtype H5, H7, and H9 viruses, including the H5N1 sublineages circulating in Eurasia and Africa (data not shown). The comparison of the results obtained from applying the conventional diagnostic method (VI) and the RRT-PCR assay to these clinical samples showed good agreement. The lowest level of agreement was observed in the RRT-PCR/VI results for subtype H5. Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival. Many of the samples that proved positive for subtype H5 by RRT-PCR but negative by VI were submitted to the OIE/FAO Reference Laboratory from Africa and the Middle East, and in some cases, the cold chain was not maintained during shipping, compromising the viability of the viruses. Based upon the results obtained in the present study, the RRT-PCR assay for simultaneous detection of subtypes H5, H7, and H9 could be a useful instrument for rapid screening and surveillance in wild and domestic birds. Although this method cannot replace the standard VI technique, this RRT-PCR assay offers several advantages over standard methods, and it could be used as a reliable tool for the rapid detection of the three AI virus subtypes, including identification of cocirculating strains. Routine application in critical environments, such as live-bird markets, or for samples obtained from wild birds in their breeding or resting sites could give an indication of the degree of coinfection with these subtypes, providing insight into the complex ecoepidemiology of AI infections in such birds.

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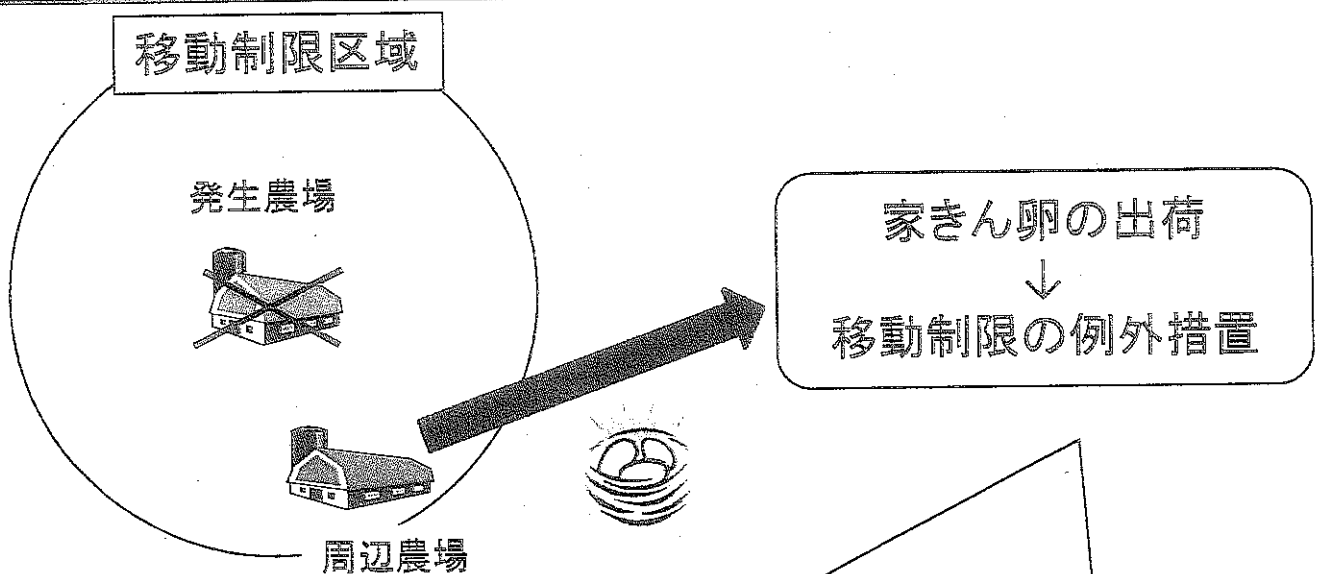
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リアルタイムRT-PCRキットの導入に係る「留意事項」(消費・安全局長通知)の一部改正について

欧州製のリアルタイムRT-PCRキットを導入、都道府県への配付(A型インフルエンザ、H5,H7亜型)



従来のRT-PCRと同様、家きん卵の出荷のための検査として位置付け



○ 例外規定を適用するためには以下の家きん卵出荷検査で陰性を確認することが条件

旧	新
<ul style="list-style-type: none"> ・臨床検査 ・ウイルス遺伝子検出検査 <ul style="list-style-type: none"> ①RT-PCR検査 ②動物用医薬品として承認された診断薬 	<ul style="list-style-type: none"> ・臨床検査 ・ウイルス遺伝子検出検査 <ul style="list-style-type: none"> ①RT-PCR検査又はリアルタイムRT-PCR検査 ②動物用医薬品として承認された診断薬

家きん卵出荷監視検査におけるウイルス遺伝子検出検査（リアルタイムRT-PCR検査）

Matrix遺伝子の保存領域を標的にしたプライマーでA型インフルエンザウイルスを検出するとともに、必要に応じて、HA遺伝子HA2領域を標的にしたプライマーでH5亜型、H7亜型の同定を行う。

1 RNA抽出

キアゲン社のRNeasy MiniKit若しくはこれと同等の能力を有するRNA抽出キットを用い、それぞれ添付マニュアルに従う。

2 リアルタイムRT-PCR検査

x-0v0社のThe FLOCKSCREEN AI-4 PCR Kit（以下「リアルタイムPCRキット」という。）を用い、以下の方法で検査を行う。なお、A型インフルエンザウイルスの検出を先に行い、陽性であった検体について、必要に応じて、H5亜型及びH7亜型のA型インフルエンザウイルスの検出を行う。

(1) プライマー・プローブ混合液の作成

凍結乾燥されたプライマー・プローブを1.2mLのRNase-free 蒸留水で融解し、プライマー・プローブ混合液とする。

(2) 陽性対照RNAの作成

凍結乾燥された不活化ウイルスを1mLのRNase-free 蒸留水で融解後、1の方法でRNAを抽出して、陽性対照RNAとする。

(3) リアルタイムRT-PCR反応

ア 以下のように試薬を1サンプル当たり20 μ Lずつチューブで調整し、反応液とする。

試薬	容量
RNase-free 蒸留水	4.8 μ L
プライマー・プローブ混合液	2.5 μ L
2 x RT-PCR master mix	12.5 μ L
酵素混合液	0.2 μ L
合計	20 μ L

イ 反応液を数秒ボルテックスした後、遠心し各20 μ Lずつ滅菌PCRチューブ又はリアルタイムPCR用96穴プレートのウェルに分注する。

ウ 反応液を分注した各チューブ又は各プレートのウェルに1により抽出したRNAを5 μ Lずつ加える。また、陽性対照には(2)の陽性対照RNAを、陰性対照にはRNase-free 蒸留水をそれぞれ5 μ Lずつ加える。

エ リアルタイムPCR機器の蛍光フィルターをFAMに設定し、以下の反応条件でリアルタイムRT-PCR反応を行う。

(ア) A型インフルエンザウイルス検出用反応 (FAM蛍光検出)

反応	温度	時間
逆転写	50℃	20分
初期変性	95℃	15分
変性	94℃	45秒
アニーリング・伸長	60℃	45秒

40サイクル

(イ) H5亜型、H7亜型のA型インフルエンザウイルス検出用反応 (FAM蛍光検出)

反応	温度	時間
逆転写	50℃	20分
初期変性	95℃	15分
変性	94℃	45秒
アニーリング・伸長	54℃	45秒

40サイクル

ただし、以下の a 及び b が認められない場合、その検査は無効とみなし、再検査を行うこと。

a 陽性対照の増幅曲線に明瞭な増幅が認められ、9から20の間のCycle threshold (Ct) 値を示すこと。

b 陰性対照の増幅曲線に閾値以上の^{いまだ}蛍光の増加が一切認められないこと。

(6) 結果の判定

以下により結果の判定を行う。

ア 明瞭で一定した蛍光の増加が認められ、Ct値が9から37の間にあった場合、陽性と判定する。

イ 閾値以上のFAMの蛍光の上昇が認められなかった場合、陰性と判定する。

ウ Ct値が9未満の場合、抽出した被検RNAをRNase-free 蒸留水で10倍希釈し、再検査を行う。

エ Ct値が37より大きく、一定しない弱い蛍光の上昇を示す場合、非特異的反応とみなし、陰性と判定する。

オ Ct値が37より大きく40未満であり、弱いながらも一定の蛍光上昇を示す場合、疑陽性とみなし、再検査を行う。

3 注意事項

(1) A型インフルエンザウイルスの検出とH5亜型及びH7亜型のA型インフルエンザウイルス

- スの検出で温度条件が異なるため、注意すること。
- (2) プローブは反応検出のためFAMで標識されているため、FAMに対するフィルターを有するリアルタイムPCR機器を使用すること。
 - (3) 検査の全過程を通して、酵素が含まれる溶液は4℃前後に保つこと。
 - (4) 1の抽出したRNAを保存する場合は、1週間程度であれば-20℃前後、長期間であれば-70℃前後で保存すること。
 - (5) 2の(1)のプライマー・プローブ混合液を保存する場合は、分注して、暗所で紫外線避け-20℃前後で保管すること。
 - (6) 2の(2)の陽性対照RNAを保存する場合は、数日間であれば4℃前後、長期間であれば-70℃前後で保存する。なお、保存の際は凍結融解の繰り返しによるRNA分解を避けるため、分注保存すること。

I-2 定点モニタリング等における新たに承認されたエライザキットの活用

高病原性鳥インフルエンザのモニタリング等に係るエライザキットの活用(イメージ)

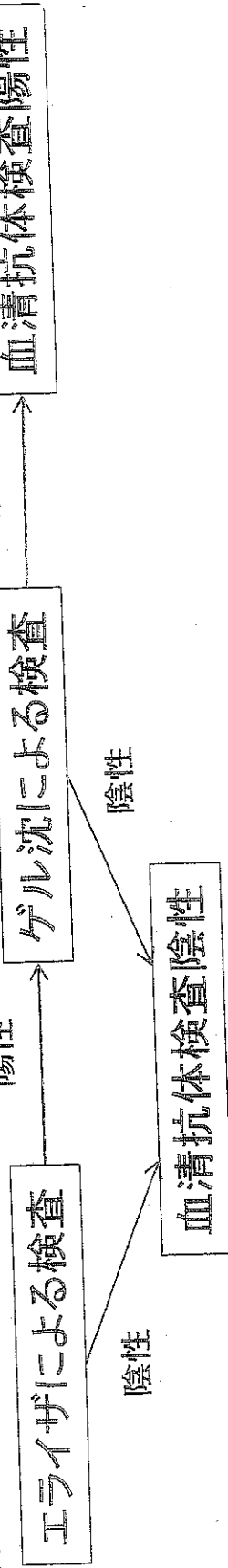
これまでのモニタリング等における血清抗体検査については、ゲル内沈降反応法で実施してきたところであるが、今般、本病に係るエライザキットが薬事法の承認を取得

○ゲル内沈降反応法(薬事法上未承認)の特徴

- ・対象家きん: 全家きん
 - ・所要時間: 48時間
 - ・活用方法: モニタリング、発生状況検査、清浄性確認検査
- ## ○エライザキットの特徴
- ・対象家きん: 鶏
 - ・所要時間: 約3時間
- (・米国では当エライザキット陽性の場合、ゲル沈での再検査で陽性の場合のみ抗体検査陽性と判定)

これまでのゲル沈による検査を引き続き実施するが、鶏の検査の場合に限り、エライザキットも選択可能とする

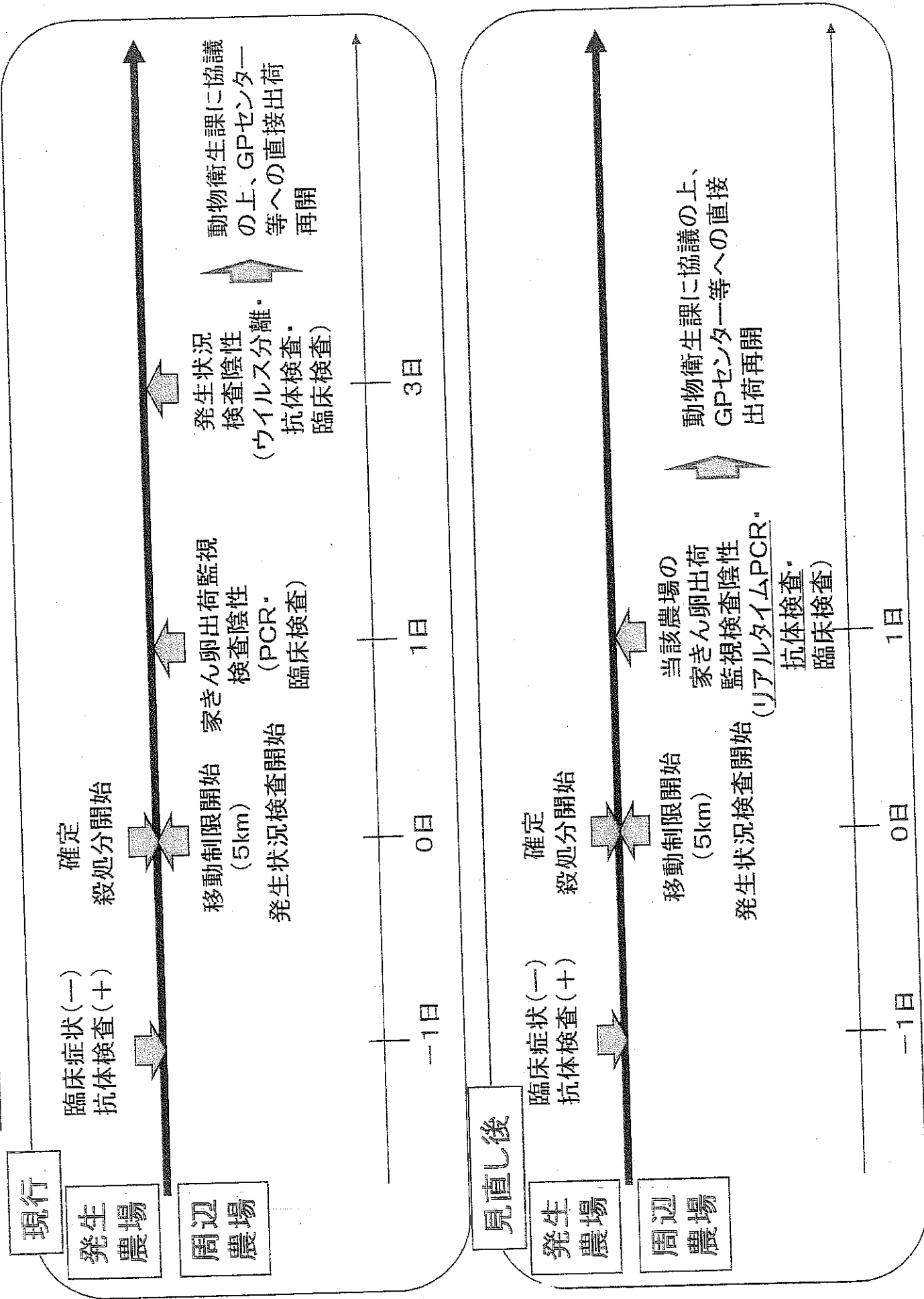
具体的な活用方法



※ OIEコードにおいては、血清学的検査法としてゲル内沈降反応法、HA法及びHI法並びにエライザ法が紹介されている

I-3 家きん卵出荷監視検査の見直しについて

弱毒タイプの発生時の家きん卵出荷検査の取扱いについて



I - 4 弱毒タイプ発生時の移動制限措置の例 外に係る確認事項の明確化

弱毒タイプの高病原性鳥インフルエンザ発生時の移動制限措置の例外に係る
確認事項の明確化について

○弱毒タイプ発生に係る例外規定（防疫指針第2の9の（3）のウ）のうち、
以下の項目については、施設のチェックリスト等の設定がされていなかったため、それぞれの確認事項を留意事項に明記

（ア）移動制限区域内の食鳥処理場の再開

→強毒タイプ発生時の移動制限区域内の食鳥処理場の再開のための
チェックリスト（別表3）により確認

（イ）移動制限区域内の食鳥処理場に直接搬入する移動制限区域内の家き
んの移動

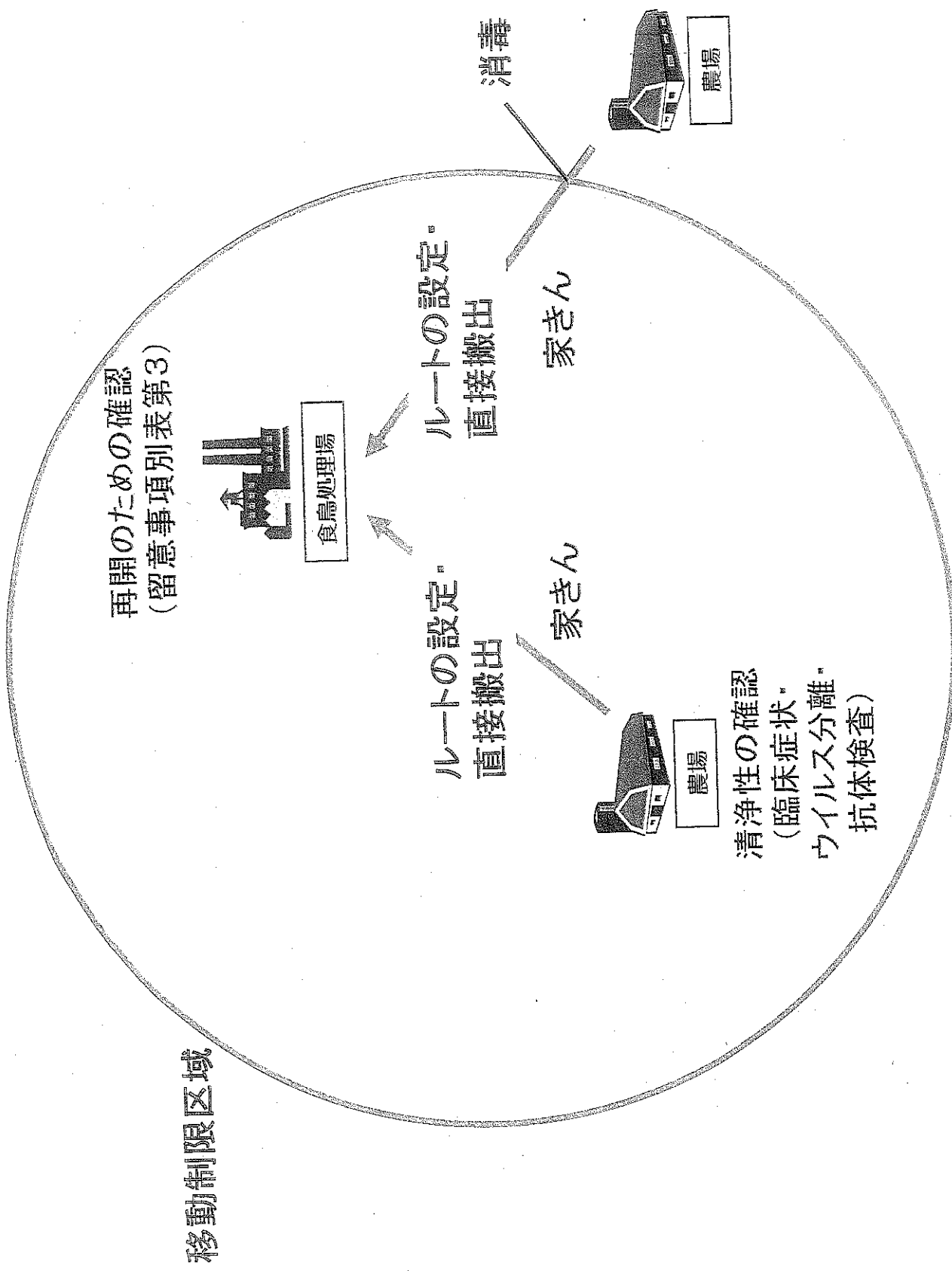
→農場が発生状況検査で陰性であり、かつ、出荷1週間以内に出荷
予定ロットから無作為に10羽以上抽出、採材（血清、気管スワ
ブ及びクロアカスワブ）し、ウイルス分離検査及び血清抗体検査
により陰性であることを確認

（ウ）移動制限区域内で生産された種卵を用いるふ卵業務の再開

→強毒タイプ発生時の移動制限区域内のふ卵場の再開のためのチエ
ックリスト（別表4（2、13、14及び15の項目を除く））
により確認

また、種卵の移動元農場については、発生状況検査及び家きん卵
出荷検査で陰性を確認

①弱毒タイプの場合の移動制限区域内の家きんの出荷



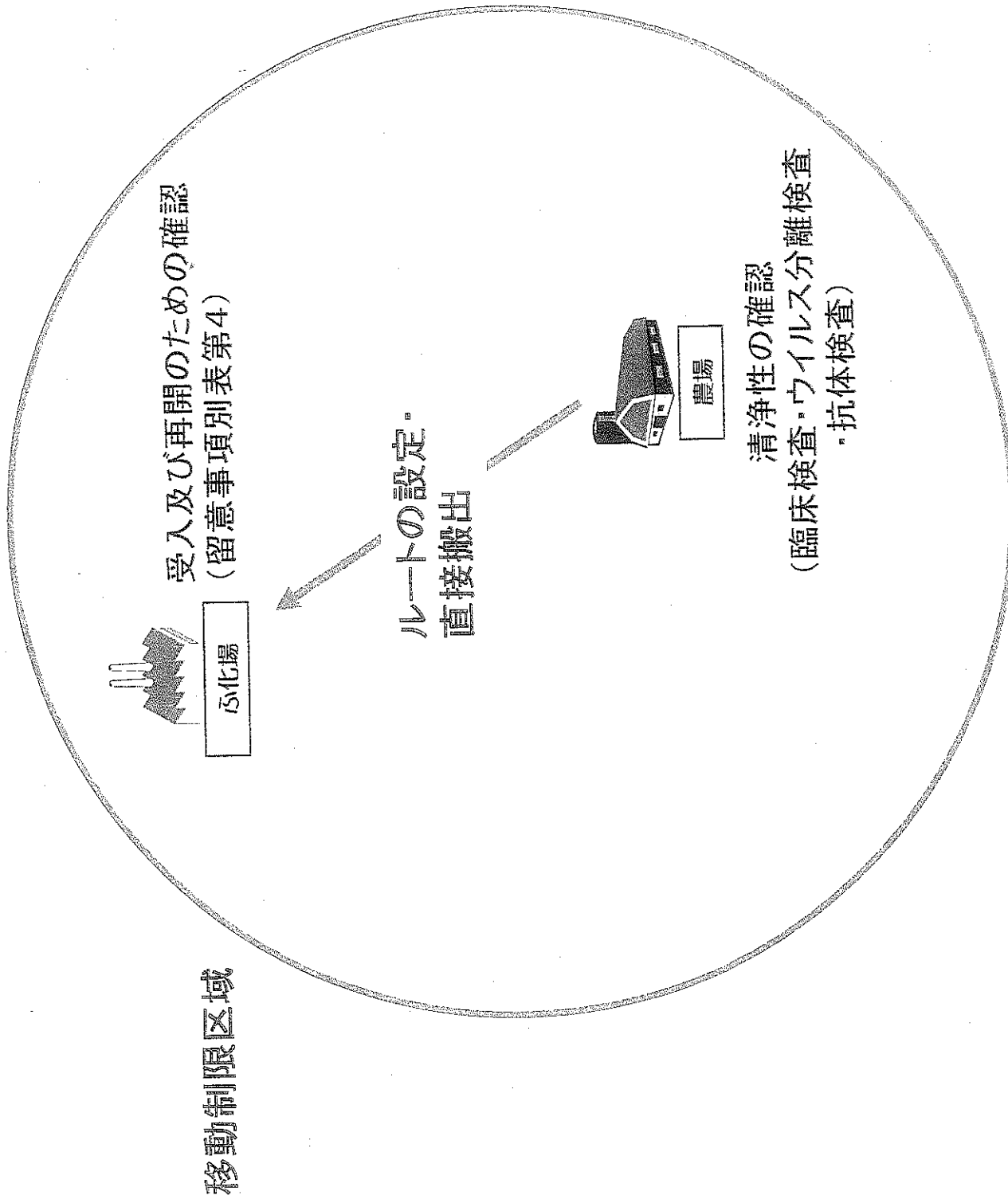
食鳥処理場の再開に当たっての確認事項

確認事項	備考
1 車両(家きん運搬、製品運搬等に使用する車両を含む。以下同じ。)及び作業従事者(関係者を含む。以下同じ。)は、入場前及び出場後、他の家きん飼養場所を含む関連施設には立ち入らないこと。	<input type="checkbox"/> 運搬ルート等の確認
2 車両は、制限区域の境界等に設けられた消毒ポイントで消毒すること。	<input type="checkbox"/> 実施記録の確認
3 車両は入出場時、消毒すること。	<input type="checkbox"/> 消毒設備の現場確認 <input type="checkbox"/> 実施記録の確認
4 作業従事者が作業場(家きん又は製品を取り扱う場所をいう。以下同じ。)に立ち入る場合には、専用の作業服、靴、帽子、手袋等を使用すること。	<input type="checkbox"/> 現場確認
5 作業場は、施設の他の場所と明確に区別され、害虫、野鳥等の侵入を防止する構造となり、又は防止する措置を講じていること。	<input type="checkbox"/> 現場確認
6 害虫が発生しないよう、作業場の内外を定期的に清掃すること。	<input type="checkbox"/> 実施記録の確認
7 移動制限区域外の家きん、出荷カゴ等の輸送に当たっては、羽毛などの飛散を防止するためシート等で遮断した車両を使用すること。	<input type="checkbox"/> 現場確認
8 使用後の出荷カゴ等は消毒し、害虫、野鳥等と接触しないような場所で保管すること。	<input type="checkbox"/> 消毒設備の現場確認 <input type="checkbox"/> 実施記録の確認
9 移動制限区域外から家きんを搬入する前に、作業場の消毒・清掃を行うこと。	<input type="checkbox"/> 現場確認
10 1から9までを含め、当該施設の特性に応じた衛生管理マニュアル等が定められていること。	<input type="checkbox"/> 衛生管理マニュアル等の確認 <input type="checkbox"/> 当該施設の平面図、家きんの搬入から製品出荷までのフロー図等の確認

1 1 1 0の衛生管理マニュアル等に基づく措置について、定期的に記録していること。	<input type="checkbox"/> 記録簿の確認
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※「備考」は、確認事項の実施・遵守状況を確認する方法。

②移動制限区域内の種卵のふ化場への直接移動



ふ卵場の再開及びひなの移動に当たっての確認事項

確認事項	備考
1 車両（種卵運搬、ひな運搬等に使用する車両を含む。以下同じ。）及び作業従事者（関係者を含む。以下同じ。）は、入場前及び出場後、他の家きん飼養場所を含む関連施設には立ち入らないこと。	<input type="checkbox"/> 運搬ルート等の確認
2 車両は、制限区域の境界等に設けられた消毒ポイントで消毒すること。	<input type="checkbox"/> 実施記録の確認
3 車両は入出場時、消毒すること。	<input type="checkbox"/> 消毒設備の現場確認 <input type="checkbox"/> 実施記録の確認
4 作業従事者が作業場（種卵又はひなを取り扱う場所をいう。以下同じ。）に立ち入る場合には、専用の作業服、靴、帽子、手袋等を使用すること。	<input type="checkbox"/> 現場確認
5 作業場は、施設の他の場所と明確に区別され、害虫、野鳥等の侵入を防止する構造となり、又は防止する措置を講じていること。	<input type="checkbox"/> 現場確認
6 作業場は、貯卵室、ふ化室、ひな処理室等が衛生的に区分された状態で設置され、種卵及びひなが接触しない構造であること。	<input type="checkbox"/> 現場確認
7 作業場の出入口には、消毒設備、踏込消毒槽、器具の洗浄場所等を設置するとともに、定期的な作業場の清掃・消毒すること。	<input type="checkbox"/> 消毒設備の現場確認 <input type="checkbox"/> 実施記録の確認
8 ふ卵に伴う残存物等（卵殻、発育停止卵、死ごもり卵、綿毛、胎便等）は焼却又は消毒後廃棄すること。	<input type="checkbox"/> 現場確認 <input type="checkbox"/> 実施記録の確認
9 種卵、コンテナ又はトレー等の輸送に当たっては、コンテナ車両等の密閉可能な車両を使用すること。	<input type="checkbox"/> 現場確認
10 使用後のコンテナ、トレー等は消毒し、害虫、野鳥等と接触しないような場所で保管すること。	<input type="checkbox"/> 現場確認 <input type="checkbox"/> 実施記録の確認
11 施設へ搬入した種卵は、入卵時及びハッチャー内でホルマリンくん蒸又はそれと同等の消毒措置を行うこ	<input type="checkbox"/> 現場確認 <input type="checkbox"/> 実施記録の確認

と。	
1 2 ハッチャーは、使用に先立って、ホルマリンくん蒸又はそれと同等の消毒措置を行うこと。	<input type="checkbox"/> 現場確認 <input type="checkbox"/> 実施記録の確認
1 3 ひなの移動に当たっては、8の(5)に定めるひな出荷監視検査ですべて陰性を確認すること。	<input type="checkbox"/> 現場確認
1 4 移動制限区域内で生産された種卵については、ふ卵の中止又はふ化後のひなの廃棄等を行い、適切に処理すること。	<input type="checkbox"/> 現場確認
1 5 ひなの輸送に当たっては、密閉可能な専用車両(トラックバン)を使用し、入場前及び出場後は、他の家きん飼養場所を含む関連施設には立ち入らないこと。	<input type="checkbox"/> 現場確認 <input type="checkbox"/> 運搬ルート等の確認
1 6 1から15までを含め、当該施設の特性に応じた衛生管理マニュアル等が定められていること。	<input type="checkbox"/> 衛生管理マニュアル等の確認 <input type="checkbox"/> 当該施設の平面図、搬入から製品出荷までのフロー図等の確認
1 7 16の衛生管理マニュアル等に基づく措置について、定期的に記録していること。	<input type="checkbox"/> 記録簿の確認

※「備考」は、確認事項の実施・遵守状況を確認する方法。