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Etude de la survie du virus H5N1 dans des environnements aquatiques artificiels reproduisant les biotopes naturels du Cambodge, pays d'endémie en zone tropicale

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Etude de la survie du virus H5N1 dans des environnements aquatiques artificiels reproduisant les biotopes naturels du Cambodge, pays d'endémie en zone tropicale

A MA CHERE MÈRE **SOEUNG VOUCH LEANG**

- Qui a fait tant de sacrifices pour l'éducation de ses enfants.
- Qui a tout sacrifié pour le bien-être et l'avenir de ses enfants
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ARN: Acide Ribonucléique

EID50: Egg Infectious Dose 50% (dose infectieuse infectant 50% des oeufs)

FAO: Food and Agriculture Organization

FP: Faiblement Pathogène

HA (H): Hémagglutinine

HP: Hautement Pathogène

IA: Influenza aviaire

IAFP: Influenza Aviaire Faiblement Pathogène

IAHP: Influenza Aviaire Hautement Pathogène

NA (N): Neuraminidase

NEP: Nuclear Export Protein

NP: Nucléoprotéine

OIE: Office International des Epizooties (Organisation mondiale pour la santé animale)

OMS (WHO): Organisation Mondiale de la santé (World Health Organization)

PA: Protéine Acide

PB1: Protéine Basique 1

PB2: Protéine Basique 2

PEG: Polyéthylène glycol

ppm: parties pour mille

RNP: Ribonucléoprotéines

RT-PCR: Reverse Transcriptase-Polymérase Chaîne Réaction

TCID50: Tissue Culture Infectious Dose 50% (dose permettant l'infection de 50 % des cellules en culture)

VIA: Virus Influenza de type A

Résumé

Bien que la persistance du virus H5N1 dans l'environement soit possible, il n'existe aucune méthode bien définie, standardisée, pour détecter du virus à partir de l'eau, du sol ou de la boue. De plus, il n'y a que très peu de données relatives au rôle du virus H5N1 dans l'environnement en pays tropical. Dans ce travail, des méthodes de concentration, d'identification, et de quantification du virus influenza dans l'eau et dans la boue ont été développées, validées puis utilisées pour l'analyse de prélèvements de l'environnement collectés au cours d'investigations d'épidémies de virus H5N1 au Cambodge et pour l'étude de la survie du virus influenza aviaire dans des biotopes aquatiques artificiels reproduisant le plus possible les conditions naturelles observées dans les mares ou les lacs au Cambodge.

L'ARN du virus H5N1 été détecté dans 19% des échantillons environnementaux de terrain collectés au décours des épidémies. Des particules virales infectieuses ont été isolées dans un échantillon d'eau d'une ferme. Dans des systèmes expérimentaux, le virus H5N1 infectieux persiste seulement 4 jours dans l'eau de pluie. Mais l'ARN viral peut encore être détecté jusqu'à 20 jours dans l'eau de pluie et 7 jours dans l'eau de mare ou de lac. Dans la boue, les particules virales infectieuses ne semblent pas pouvoir survivre bien que l'ARN puisse persister au moins 2 semaines. La faune et la flore aquatique n'ont aucune influence sur la persistance du virus infectieux dans l'eau. Ces organismes semblent être essentiellement des concentrateurs et des transporteurs passifs du virus plutôt que des hôtes autorisant la réplication du virus. Nos résultats montrent que l'environnement au cours d'épidémies est fortement contaminé par le virus H5N1 et pourrait constituer une source potentielle de contamination humaine et/ou animale. Une restriction de l'accès à l'eau potentiellement contaminée doit être recommandée autour des foyers épidémiques. La surveillance de l'environnement doit être intégrée dans les programmes de lutte contre la grippe aviaire qui doivent par conséquent prendre en considération des mesures de désinfection de l'environnement.

Mots clés : virus H5N1, environnement, eau, boue, biotopes aquatiques, Cambodge.

Summary

Although the persistence of the H5N1 virus in the environment is possible, there is no well-defined and standardized method for the detection of viruses from water, soil or mud. In addition, there is very little data available regarding the role of H5N1 virus in the environment in the tropics. In this work methods of concentration, identification, and quantification of influenza viruses in water, mud and soils have been developed, validated and used to test environmental samples collected following H5N1 outbreaks in Cambodia and to analyze samples obtained during experiments in artificial aquatic biotopes aiming to reproduce as faithfully as possible the characteristics observed in ponds and lakes in Cambodia. The H5N1 viral RNA was detected in 19% of environmental samples. Among these, infectious viral particles were isolated in a single water sample. In experimental systems, the infectious H5N1 virus survived only 4 days in the rain water. But viral RNA persisted up to 20 days in rain water and 7 days in pond and lake water. In mud, infectious viral particles did not survive even viral RNA could persist for at least 2 weeks. Aquatic flora and fauna have no influence on infectious H5N1 virus persistence in water. These organisms seem to concentrate and to passively carry the virus but do not allow virus replication. Our results showed that following outbreaks, the environment is widely contaminated by H5N1 virus and therefore can act as a potential source of human and/or animal contamination. Restricted access to potentially contaminated water should be recommended during outbreak episodes. Monitoring the environment is recommended in the effort to fight against avian influenza and measures including environment disinfection should also be considered.

Key words: H5N1 virus, environment, water, mud, aquatic biotopes, Cambodia

Introduction générale

Les virus influenza de type A (VIA) font partie de la famille des *Orthomyxoviridae*. Ce sont des virus à ARN monocaténaire enveloppés. Tous les sous-types du VIA (H1 à H16 et N1 à N9) ont été isolés chez des oiseaux aquatiques sauvages (Munster et al., 2007; Webby et al., 2007). Mais les canards sont les principaux réservoirs naturels et généralement les porteurs asymptomatique du virus (Webster et al., 1992). Le virus de l'influenza aviaire se réplique dans le tractus respiratoire et gastro-intestinal. Les oiseaux infectés excrètent en grande quantité du virus dans leurs fèces, salive et déjections nasales (Swayne and Halvorson, 2003; Webster et al., 1978). Cela conduit à la contamination de matériaux de l'environnement, tels que l'eau, les sédiments aquatiques, le sol, la boue (Ito et al., 1995; Lang et al., 2008; Pannwitz et al., 2009). Webster et collaborateurs (1992) ont montré que la transmission des virus de l'influenza aviaire est réalisée par voie féco-orale, par l'utilisation partagée d'une source commune d'eau contaminée pourrait facilitant la dissémination du virus.

Le virus influenza H5N1, responsable de l'Influenza Aviaire Hautement Pathogène (IAHP), s'est répandu en Asie puis dans le reste du monde, causant des pertes économiques et de nombreuses inquiétudes quant au risque pandémique associé. La progression et la réapparition de la grippe aviaire posent de nombreuses questions quant à sa transmission et son éventuelle survie dans l'environnement. Il a été rapporté que le virus influenza H5N1 était excrété en plus grande quantité et pour une plus longue durée dans la trachée et les voies respiratoires supérieures par rapport à la voie cloacale (Brown et al., 2006; Henaux and Samuel, 2011; Webster et al., 1992). La persistance du virus H5N1 dans l'eau a également été démontrée (Brown et al., 2007b; Domanska-Blicharz et al., 2010). La plupart des cas d'infection humaine sont liés au contact rapproché avec des volailles infectées: l'inhalation d'aérosols de gouttelettes infectées est probablement la voie préférentielle d'infection (Brankston et al., 2007). Cependant l'ingestion d'eau contaminée pourrait être un possible

mode de contamination humaine (WHO, 2007). Bien que la persistance du virus H5N1 dans l'environement soit possible (de Jong et al., 2005; Iglesias et al., 2010; Lebarbenchon et al., 2010; Vong et al., 2008; Vong et al., 2009; WHO, 2007), il n'existe aujourd'hui aucune méthode bien définie, standardisée, pour extraire et détecter du virus à partir de l'eau, du sol ou de la boue. De plus, il n'y a que très peu de données sur l'écologie du virus H5N1 hautement pathogène (HP) dans l'environnement naturel bien que ce dernier puisse potentiellement contribuer à la transmission du virus (WHO, 2007). Brown et al. (2007b) ont suggéré que le virus IAHP pourrait être moins apte que les virus influenza type A faiblement pathogènes (IAFP) à diffuser dans l'eau via la voie féco-orale. Cependant la persistence du H5N1 HP dans l'eau, la boue ou le sol de l'environnement n'a pas été bien documentée.

Au Cambodge, depuis la première détection du virus H5N1 HP en 2004, 18 cas humains d'infection à H5N1 et presque 30 épizooties du H5N1 chez les volailles ont été rapportées (OIE, 2011; WHO, 2011a). Les cas humains apparaissent surtout après contact direct avec des volailles infectées (Buchy, 2008; Buchy et al., 2007) bien que plusieurs études séro-épidémiologiques ont établi que de se laver et de nager dans les mares constituait un risque de contamination humaine (infections asymptomatiques) (Cavailler et al., 2010; Vong et al., 2009). De plus, dans une autre étude réalisée au Cambodge, après la confirmation d'un cas humain d'infection à virus H5N1 en 2006, des échantillons d'eau et de boue ont montré des résultats positifs en RT-PCR quantitative en temps réel (qRT-PCR) (Vong et al., 2008). Cependant le rôle exact de l'environnement dans la transmission du virus H5N1 reste mal compris. Et très peu d'informations sont disponibles concernant la persistance du virus H5N1 dans l'environnement durant les épidémies dans les pays tropicaux.

La capacité des virus à résister dans l'environnement est variable entre virus de sous-types différents, mais également entre virus de même sous-type. Différents paramètres physico-chimiques comme la température, la salinité et le pH jouent un rôle important dans la survie du virus influenza dans l'environnement (Brown et al., 2007b; Stallknecht and Brown, 2009; Stallknecht et al., 1990b; Zarkov, 2006). La composition physico-chimique mais aussi la

présence ou l'absence de micro-organismes dans l'eau revêtent donc une importance capitale quant à la survie du virus influenza. En dehors des effets des composants abiotiques du milieu aquatique, on connaît peu l'influence des facteurs biologiques sur la persistance du VIA dans l'eau. Il n'y a également que peu d'informations sur la capacité des animaux aquatiques vivant dans l'eau potentiellement contaminée par des VIA à se contaminer et en retour à transmettre le virus dans l'eau et aux autres animaux aquatiques. Il est donc intéressant d'étudier la survie du virus H5N1 dans un système expérimental recréant un environnement aquatique artificiel particulièrement dans les conditions reproduisant le plus fidèlement possible l'environnement aquatique naturel en pays tropical comme le Cambodge.

Ce travail de thèse a pour but de répondre aux objectifs suivants :

1) Valider les techniques de concentration, d'identification et de quantification du virus influenza H5N1 dans l'eau et dans la boue

2) Observer la survie du virus influenza H5N1dans l'environnement naturel;

3) Observer la survie du virus H5N1 dans des systèmes expérimentaux visant à recréer des environnements aquatiques artificiels simples et complexes.

PREMIERE PARTIE : virus influenza A et virus H5N1

1. Généralités sur les virus influenza A

1.1. Classification – propriétés

Les virus influenza A appartiennent à la famille des *Orthomyxoviridae* qui inclut 4 genres: les influenzavirus A, les influenzavirus B, les influenzavirus C et les thogotovirus. Il s'agit de virus à ARN de polarité négative et segmentés. La distinction entre les types de virus influenza A, B et C est fondée sur l'antigénicité de la nucléoprotéine (NP). Les virus influenza A se subdivisent de plus en de nombreux sous-types à partir de leurs antigènes de surface: l'hémagglutinine (HA) et la neuraminidase (NA). L'hémagglutinine permet l'attachement du virus à un récepteur cellulaire de l'hôte par reconnaissance des acides sialiques que ce récepteur porte, tandis que la neuraminidase permet l'hydrolyse du récepteur cellulaire, ce qui favorise la libération des particules virales. Il existe chez les virus influenza A 16 hémagglutinines (H1-H16) et 9 neuraminidases (N1-N9) différentes (Palese and Shaw, 2007; Wright et al., 2007). Toutes les combinaisons (HxNy) entre ces protéines de surface sont théoriquement possibles. Dans ce travail, nous avons pris comme modèle le virus H5N1 d'origine aviaire (Gutiérrez et al., 2009).

Les *Orthomyxoviridae* sont des virus enveloppés, mesurant entre 80 et 120 nm de diamètre habituellement de forme globalement sphérique ou parfois filamenteuse (Figure 1A). L'enveloppe dérive de la membrane cytoplasmique des cellules hôtes et héberge les deux glycoprotéines d'enveloppe, à savoir la HA et la NA, ainsi que la protéine M2. La face interne de l'enveloppe est tapissée par la protéine de matrice M1 (Figure 1B). Les particules virales renferment le génome viral composé de 8 segments d'ARN monocaténaire de polarité négative. Associés à la nucléoprotéine (NP) ainsi qu'aux trois protéines PB1 (protéine basique 1), PB2 (protéine basique 2) et PA (protéine acide) qui forment le complexe polymérase, ils se présentent sous la forme de ribonucléoprotéines (RNP) de structure

hélicoïdale d'un diamètre de 9 nm. La particule virale renferme en outre la protéine NEP (Nuclear Export Protein) précédemment nommée NS2.

Figure 1 : structure du virus influenza A.

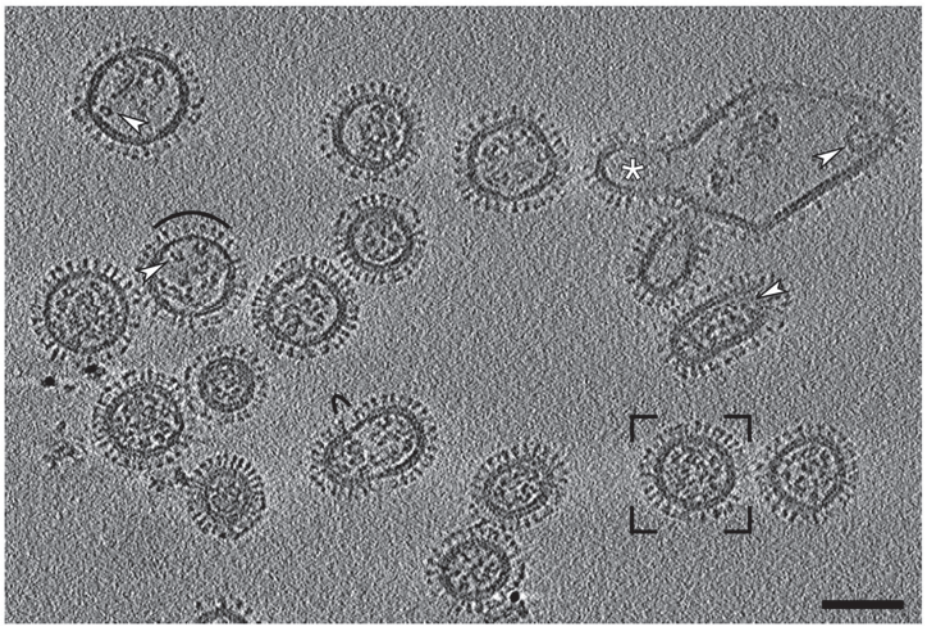


Figure 1A: ultra-structure de virus influenza par tomographie cryoélectronique. Les flèches blanches montrent les ribonucléoprotéines. D'après (Harris et al., 2006)

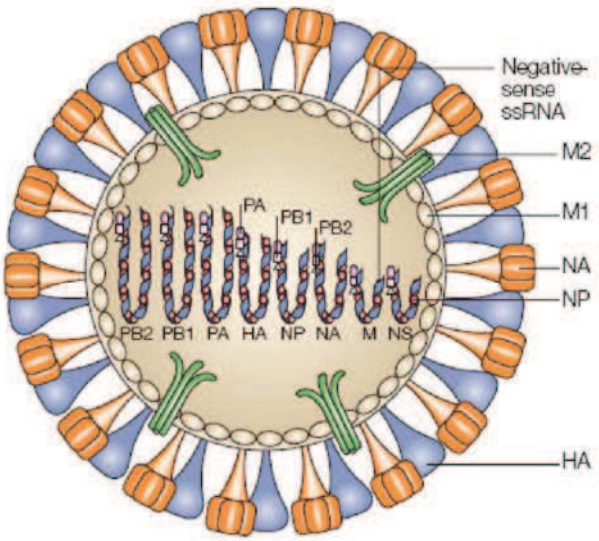


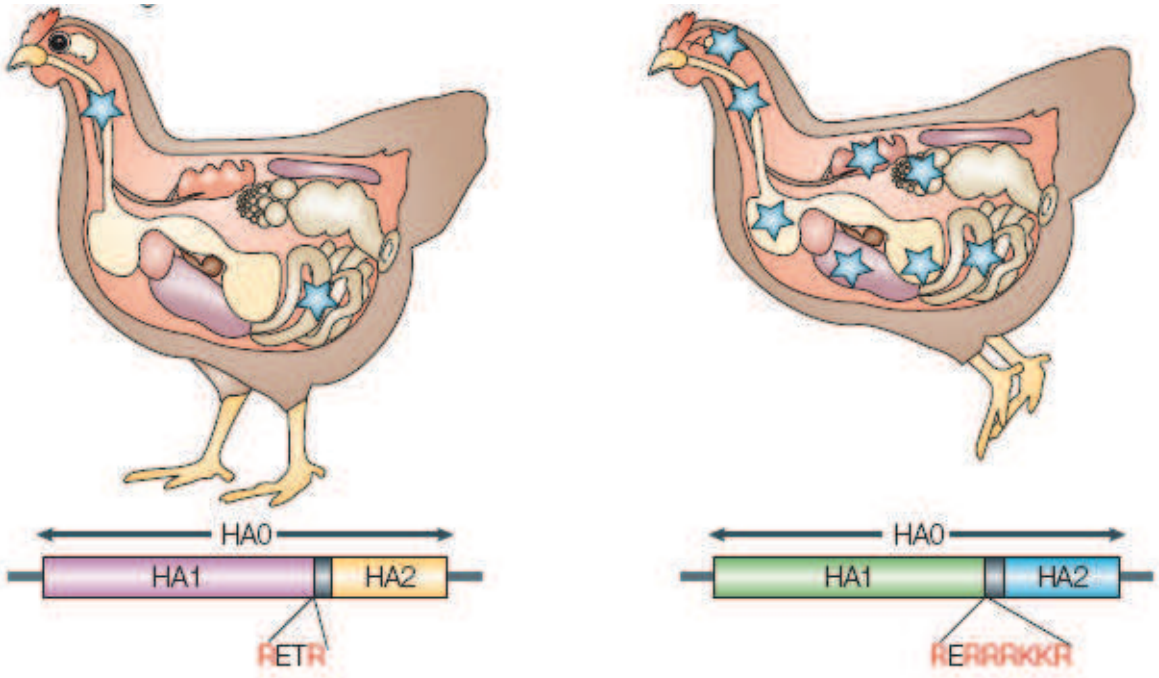
Figure 1B: représentation schématique de la structure du virus influenza A : les protéines de surface HA, NA et M2 sont insérées dans la bicouche lipidique de l'enveloppe virale. La protéine de matrice M1 tapisse la face interne de l'enveloppe. Les 8 segments d'ARN de polarité négative, associés à la NP et aux protéines PB1, PB2 et PA du complexe de transcription/réplication, forment les ribonucléoprotéines (source: (Horimoto and Kawaoka, 2005)

1.2. Pathogénie

Il existe de grandes variations de degré de pathogénicité dans les influenzavirus de type A. On peut ainsi les diviser en deux pathotypes en fonction du degré de pathogénicité chez les espèces aviaires d'élevage. Le premier pathotype, l'influenza aviaire hautement pathogène est responsable d'infections systémiques et mortelles. Cette catégorie ne comprend à ce jour que des virus influenza de sous-type H5 et H7. La grippe aviaire hautement pathogène est caractérisée par un début brutal, une maladie sévère, de propagation rapide, et un taux de mortalité s'approchant de 100% dans les 48 heures suivant l'infection (WHO). La seconde catégorie, l'influenza aviaire faiblement pathogène, est responsable en général d'infections inapparentes ou bénignes (Alexander, 2007).

La protéine HA joue un rôle crucial dans la pathogénicité des virus de la grippe aviaire. Les liens ont clairement été établis entre la virulence et la capacité de coupure de cette protéine HA dépendant de la présence ou non de plusieurs acides aminés basiques au niveau de ce site de clivage (Horimoto and Kawaoka, 1994, 2001; Klenk and Rott, 1988). Les virus hautement pathogènes des sous-types H5 et H7 possèdent ce motif de plusieurs acides aminés basiques et leur HA peut ainsi être clivé au niveau intracellulaire par des protéases ubiquitaires. Ainsi, ces virus ont la capacité de provoquer des infections systémiques chez les volailles. Les virus peu pathogènes, à l'exception du virus H7N7 équin, ne possèdent qu'une arginine au site de clivage de la HA et ainsi ne peuvent se multiplier qu'au niveau des quelques organes où la protéase nécessaire au clivage de la protéine HA est présente (Bosch et al., 1979). Les virus aviaires FP n'entraînent, par conséquent, que des infections localisées, généralement limitées à l'arbre respiratoire et/ou au tractus digestif, et peu ou pas symptomatiques. Le tropisme tissulaire des virus est ainsi, au moins partiellement, déterminé par la présence de protéases de l'hôte capables ou non de reconnaître et de cliver la HA au niveau d'un site comportant soit 1 seul soit plusieurs acides aminés à caractère basique.

Figure 2: rôle du site de clivage dans la pathogénicité des virus influenza aviaires



Le clivage de du précurseur de la HA (HA0) en sous-unités HA1 et HA2 par les protéases de l'hôte libère un domaine de fusion (en gris) au niveau de la partie amino-terminale de la HA2 qui permet la fusion entre la membrane virale et la membrane de l'endosome. Ainsi, l'activité protéolytique est essentielle pour l'infectivité du virus. La HA des virus peu pathogènes (A) ne contient pas de série d'acides aminés basiques au niveau du site de clivage et est clivée par des protéases localisées dans le système respiratoire et le tube digestif (étoiles bleues) ce qui se traduit par des infections modérées et localisées. La HA des virus hautement pathogènes (B) possède un motif de multiples acides aminés basiques au niveau du site de clivage et peut ainsi être clivée par des protéases ubiquitaires présentes dans de nombreux tissus et organes (étoiles bleues) ce qui entraîne une infection systémique létale. D'après Horimoto et Kawaoka, 2005.

1.3. Epidémiologie

Les oiseaux sont sensibles aux infections par les virus de type A, cependant certaines espèces d'oiseaux (oiseaux sauvages aquatiques) peuvent abriter ces virus de manière asymptomatique. D'autres espèces d'oiseaux y compris les oiseaux d'élevage (poulets, dindons, cailles, pintades, etc.) ainsi que les oiseaux d'ornement et les oiseaux sauvages non aquatiques sont sensibles à ces virus et peuvent développer la maladie. Certaines souches entraînent un taux de mortalité particulièrement élevé. Le virus H5N1 a également été isolé chez des mammifères infectés naturellement ou expérimentalement dont l'homme, le rat, la souris, le vison, le furet, le porc, le chat, le tigre, le chien, etc. (OIE, 2008).

Chez les volailles domestiques, la propagation de la forme AIHP est très rapide et la maladie est ainsi hautement transmissible causant une forte mortalité chez les oiseaux. Les oiseaux

infectés excrètent le virus en grande quantité dans leurs fèces mais aussi dans les sécrétions nasales et oculaires (Swayne and Halvorson, 2003). La propagation virale est un phénomène continu chez les oiseaux sauvages aquatiques qui sont le réservoir naturel du virus. Des mesures ont été prises pour contrôler les épidémies de grippe aviaire hautement pathogène à virus H5N1 et plusieurs centaines de millions de volailles ont été abattues afin de contrôler l'épizootie. Malgré cela, les épidémies continuent à réapparaître et il semble que le virus H5N1 soit devenu endémique dans plusieurs pays d'Asie. Depuis la fin 2005, l'épizootie a lentement progressé vers l'ouest en Eurasie, puis vers l'Europe en novembre 2005 et enfin en Afrique début 2006 (WHO, 2011b). Depuis 2003, les vagues d'épidémie observées sont plus graves et plus importantes.

En dehors du problème de santé animale, cette épizootie globale est associée aussi à un problème de santé humaine. A ce jour, 566 cas humains d'infection par le virus H5N1 ont été rapportés et 332 de ces cas sont décédés(WHO, 2011a). Tous les cas humains sont apparus dans les pays où il existe des épidémies de AIHP chez les volailles. La plupart des cas humains pourraient être liés au contact avec les volailles infectées, mais des cas de transmission interhumaine ont également été suggérés (Peiris et al., 2007). Le passage rare de ce virus zoonotique des oiseaux vers l'homme pose la menace d'émergence du virus hautement pathogène, qui pourrait s'adapter plus efficacement à l'homme et déclencher une pandémie.

1.4. Réservoir du virus influenza A dans la nature

Les virus de la grippe aviaire ont été isolés à partir de plus de 105 espèces d'oiseaux sauvages appartenant à 13 ordres différents (Olsen et al., 2006). Le réservoir naturel des virus influenza de type A comprend les oiseaux aquatiques appartenant à l'ordre des Ansériformes (oies, canards, cygnes, etc.) et des Charadriiformes (mouettes, sternes, chevaliers, etc.) (Munster et al., 2007; Stallknecht and Brown, 2008). Les virus influenza A ont également été isolés chez des espèces domestiques telles que les dindes, poulets, cailles, faisans, oies et canards. De

plus le virus H5N1 été retrouvé chez des passereaux, des pigeons et des faucons (FAO, 2005; Peiris et al., 2007).

Chez les canards, les virus aviaires se répliquent habituellement surtout au niveau des cellules épithéliales du tractus digestif et, dans une moindre mesure, au niveau de l'arbre respiratoire (Hinshaw and Webster, 1982; Webster et al., 1978). Webster et al. (1978) a rapporté que des canards infectés expérimentalement excrètent 6,4 g de matières fécales par heure, avec une dose infectieuse de $1 \times 10^{7.8}$ EID50, et ces oiseaux excrètent environ 1×10^{10} EID50 du virus IA dans un délai de 24 heures. En plus d'un haut niveau d'excrétion virale, la durée de l'excrétion virale chez des canards pourrait être prolongée. Hinshaw a rapporté que des canards infectés étaient capables d'excréter par voie digestive du virus pendant plus de 28 jours (Hinshaw et al., 1980). L'excrétion des virus IAFP est principalement fécale (Henaux and Samuel, 2011; Kida et al., 1980; Webster et al., 1978). Cependant, l'excrétion par voie orale et nasale est possible (Sturm-Ramirez et al., 2005; Swayne and Halvorson, 2003). Pour les virus IAHP, une excrétion trachéale est prédominante, même si la voie fécale existerait en parallèle, mais dans des proportions moindres (Brown et al., 2007a; Henaux and Samuel, 2011; Hulse-Post et al., 2005). Une étude expérimentale a montré que des canards infecté par le virus IAFP excrètent du virus par voie orale et cloacale en plus grande concentration et pendant une plus longue durée par rapport aux canards infectés par le virus IAHP (Henaux and Samuel, 2011). Cela signifie que les virus aviaires sont bien adaptés aux canards et que, à l'exception du virus H5N1, ils ont atteint un état de stase évolutive chez les oiseaux aquatiques sauvages. L'infection des canards est habituellement asymptomatique, bien qu'il y ait quelques exceptions (Sturm-Ramirez et al., 2005). Les oiseaux infectés excrètent les virus en grande quantité dans leurs fientes (Kida et al., 1980; Webster et al., 1978) si bien que des virus influenza A ont pu être isolés dans l'eau des lacs où les oiseaux nidifient et se rassemblent (Hinshaw et al., 1979; Ito et al., 1995). L'eau et les excréments contaminés sont, par conséquent, des voies de contamination majeures pour les oiseaux sauvages.

1.5. Les virus influenza A dans l'environnement

1.5.1. Transmission du virus influenza A

La transmission du virus influenza aviaire au sein des populations oiseaux sauvages dépend de la transmission féco-orale par l'eau contaminé (Hinshaw et al., 1979, 1980; Sandu and Hinshaw, 1981; Sinnecker et al., 1983). La transmission directe d'un oiseau à un autre par contact direct ou via des aérosols ou un support humain est donc indéniable (Webster et al., 2002). Cependant une transmission indirecte via l'environnement est également envisagée (Breban et al., 2009; Rohani et al., 2009; Vong et al., 2009; Ward et al., 2008). Concernant la transmission du virus à des mammifères, un contact rapproché et prolongé avec des oiseaux infectés s'avère nécessaire à la contamination. Les canards domestiques jouent alors un rôle important puisque ils sont porteurs du virus mais n'expriment pas souvent la maladie (porteurs asymptomatiques). En outre, une épidémie d'influenza à H5N1 touchant des petits élevages familiaux de poulets dans des pays en développement est une telle catastrophe économique pour les éleveurs, qu'il peut arriver que ces derniers abattent et vendent les quelques individus survivants sans se soucier de savoir s'ils sont porteurs du virus ou non. Le virus H5N1 HP n'a pas encore démontré la capacité de se transmettre efficacement de personne à personne malgré que quelques cas de transmission interhumaine aient été suggérés (Gutiérrez et al., 2009; Peiris et al., 2007; Van Kerkhove et al., 2011; Wang et al., 2008). Les cas humains de H5N1 hautement pathogène se sont produits principalement après un contact direct avec des volailles infectées (Areechokchai et al., 2006; Buchy, 2008; Buchy et al., 2007; Dinh et al., 2006). De plus, la transmission par l'exposition à un environnement contaminé (eau souillée, carcasses de volailles, engrais à base des excréments, litières contaminées,..) sans contact direct avec des oiseaux infectés a également été suggérée (Cavailler et al., 2010; de Jong et al., 2005; Food and Agriculture Organization; Kandun et al., 2010; Vong et al., 2009; WHO). Les connaissances sur le rôle de l'environnement dans la transmission indirecte du virus sont très limitées. Ainsi le rôle de l'eau dans le cycle

épidémiologique de l'IAFP est encore incertain et concernant l'IAHP les données sont encore plus restreintes. De plus la majorité des études ayant été menées en climat tempéré. Des efforts restaient donc à faire pour clarifier la place de l'eau et l'environnement aquatique dans le cycle naturel de la maladie en climat tropical ou subtropical.

1.5.2. La stabilité des virus de l'influenza aviaire dans l'eau

Des virus influenza aviaires ont été isolés à partir des eaux de surface en Alberta (Hinshaw et al., 1980), Minnesota (Halvorson et al., 1985), et Alaska (Ito et al., 1995) dans des habitats fréquentés par des canards sauvages. Dans certains cas, des virus IA ont été isolés à partir d'échantillons d'eau sans concentration préalable des prélèvements.

Malgré l'importance reconnue de la transmission féco-orale et hydrique de ces virus dans les populations d'oiseaux, les données existantes sur la persistance des virus IA dans les fèces, l'eau, les surfaces de l'environnement, et les carcasses sont limitées. La persistance environnementale des virus IA a été initialement étudiée par Webster et al. (1978) en utilisant la souche H3N2 (A/Duck/Memphis/546/74). Avec une dose initiale de $10^{6.8}$ EID50 dans les matières fécales et de $10^{8.1}$ EID50 dans l'eau, le virus restait infectieux pendant au moins 32 jours suggérant que le milieu aquatique contaminé pourrait constituer une source d'infection. Par la suite, la persistance du virus IA été évaluée dans les fèces (Beard et al., 1984; Lu et al., 2003) et dans liquide allantoïde (Lu et al., 2003). D'autres études (Brown et al., 2009; Brown et al., 2007b; Nazir et al., 2010; Stallknecht et al., 1990b; Stallknecht et al., 1990a) ont évalué expérimentalement la persistance de virus AIFP isolés à partir des canards sauvages dans l'eau. Collectivement, ces études expérimentales ont démontré que ces virus IA peuvent persister pendant des mois dans l'eau à 4 ° C, 17 ° C et 28 ° C. La durée de l'infectiosité était inversement proportionnelle à la température de l'eau, certains virus étant restés infectieux plus d'une année à 4 ° C, mais seulement quelques jours à 37 ° C (Brown et al., 2009). Ces études ont également déterminé que l'infectiosité du VIA est dépendante des paramètres physicochimique de l'eau (pH et salinité) à des valeurs habituellement rencontrées dans les

eaux de surface. Selon les souches, des variations phénotypiques dans la capacité à rester infectieux dans des conditions de pH variables et en milieu salin ont été observées, ainsi qu'un effet interactif entre salinité et pH (Stallknecht et al., 1990b). Le pH affecte grandement l'infectiosité, avec une diminution rapide à pH inférieur à 6,5. Les virus sont le plus stable entre pH 7,4 et pH 8,2, mais une variation de la tolérance au pH a été observée selon les virus. Du point de vue de la salinité, les virus IA sont le plus stable à 0 parties pour mille (ppm) (eau douce) ou 15 000 ppm (eau saumâtre) plutôt que 30 000 ppm (eau de mer) de chlorure de sodium. Des variations existent cependant selon les isolats. D'une manière générale la durée de survie VIA est inversement proportionnelle à la température et à la salinité de l'eau, et il existe une interaction forte entre ces deux paramètres, à savoir que plus la température baisse, plus l'effet de la salinité est délétère pour la survie du virus (Brown et al., 2007b; Zarkov, 2006). Le virus H5N1 qui paraît moins résistant que les autres virus de grippe aviaire dans l'environnement, aurait une résistance similaire aux autres virus de grippe hautement pathogènes dans les eaux salées (Brown et al., 2007b). Dans l'étude de Zarkov et al., la survie de sous-types de VIA dans l'eau dépend du titre et n'a pas dépassé 24 heures à pH 9,34, a atteint une semaine dans les conditions de salinité les plus élevées, et plus de deux semaines dans les conditions proches de celles rencontrées dans l'eau potable (Zarkov, 2006). En résumé, la survie du virus influenza aviaire dans l'eau est dépendante d'une part de la souche virale et de son titre, et d'autre part de la salinité, du pH et de la température de l'eau. En dehors de ces paramètres principaux, la présence d'une flore microbienne réduirait le temps de survie de certains virus de grippe dans l'eau (Brown et al., 2007b; Stallknecht et al., 1990a; Zarkov, 2006). L'intensité de l'exposition aux rayons ultra violets jouerait également un rôle important dans la réduction du pouvoir infectieux des virus influenza A (Sagripanti and Lytle, 2007). Le fumier contenant les fientes de volailles est traditionnellement utilisé comme engrais dans l'agriculture en Asie. Dans les fientes fraîches et humides, le virus H5N1 peut survivre jusqu'à 4 jours alors que dans un fumier desséché (14 % d'humidité) le

virus est détruit en moins de 24 heures à une température de 25° C et en 15 minutes lorsque la température du fumier atteint 40° C (Chumpolbanchorn et al., 2006; Songserm et al., 2006).

1.5.3. Détection du virus influenza aviaire dans l'environnement

Relativement peu d'études ont cherché à isoler le VIA directement à partir des eaux de surface, mais la présence de ces virus dans des échantillons environnementaux a été montrée (Halvorson et al., 1985; Hinshaw et al., 1980; Ito et al., 1995; Lang et al., 2008; Leung et al., 2007). La méthodologie utilisée dans ces études varie et à l'heure actuelle il n'existe pas de méthode unique recommandée pour isoler ou détecter ces virus dans des échantillons environnementaux. À ce jour, le VIA a été détecté avec succès dans des échantillons d'eau par mise en culture directe (Halorson et al., 1983) et après concentration avec des érythrocytes de poulet fixé préalablement au formol (Ito et al., 1995; Khalenkov et al., 2008). Le VIA a également été détecté par PCR dans des échantillons de sédiments et d'eau (Dovas et al., 2010; Lang et al., 2008; Vong et al., 2008). La détection à long terme du virus IA dans des habitats aquatiques suite au départ des oiseaux aquatiques sauvages a été rapportée dans deux études en Alaska et dans les deux cas, une prévalence relativement élevée de l'eau infectée (1% à 7% par isolement du virus) (Ito et al., 1995) ou des sédiments (56% des échantillons positifs en PCR) ont été rapportés (Lang et al., 2008).

2. Le virus H5N1 hautement pathogène

Chez les poulets, les virus influenza peuvent être responsables d'une mortalité atteignant 100% et sont caractérisés par une multiplication systémique se traduisant par des lésions au niveau de multiples organes. Si le virus H5N1 HP a causé des épidémies chez un grand nombre d'oiseaux sauvages (Ellis et al., 2004; Liu et al., 2005), il a depuis 1997, provoqué de nombreuses flambées d'infection chez les volailles en Asie du Sud-Est (Buchy et al., 2009; Chen et al., 2006; Li et al., 2004) qui ont dévasté l'industrie de la volaille dans de grandes parties de cette région depuis 2004. Il est fort probable que les oiseaux migrateurs comme les

volailles infectées ont contribué aux épidémies de virus H5N1 HP (Chen et al., 2005; Chen et al., 2006). Ces épidémies se sont accompagnées par la transmission occasionnelle du virus H5N1 à des humains, entraînant au total 566 cas humains, et conduisant à 332 décès (en date du 10 octobre 2011)(WHO, 2011a). Le virus H5N1 hautement pathogène est devenu endémique dans les populations de volailles en Asie du sud-est ainsi qu'en Egypte et a affecté au total plus de 60 pays dans le monde, y compris le Cambodge.

2.1. Importance de l'étude de l'environnement pour la compréhension de l'épidémiologie du virus H5N1.

A ce jour, l'hypothèse de la transmission directe du virus influenza d'un hôte à l'autre est largement privilégiée et donc très étudiée. Cependant, même si cette voie de transmission est indéniable, elle ne suffit pas à expliquer entièrement les faits constatés. En effet, les cas sont parfois espacés de plusieurs mois, voir de plusieurs années, ce qui implique que le virus subsiste quelque part entre deux bouffées épidémiques.

Selon Rohani *et al.,* l'environnement jouerait un rôle prépondérant dans la survenue de nouveaux foyers, mais aussi dans la persistance d'une épidémie dans le temps. Si le pic de la courbe épidémique reste presque exclusivement lié à la transmission directe, la phase ascendante initiale ainsi que la pente descendante de la courbe reposent en grande partie sur la transmission indirecte (Rohani et al., 2009).

L'exposition au virus H5N1 par des environnements contaminés peut expliquer une partie des cas d'infection (de Jong et al., 2005; Vong et al., 2008; Vong et al., 2009; WHO, 2007). L'utilisation de fèces contaminées comme engrais de jardin a été signalée comme une source d'infection humaine (Kandun et al., 2010). Les oiseaux apportant de fortes concentrations de virus dans les sources d'eau, la transmission aux humains par de l'eau contaminée est également possible (WHO, 2007). L'enquête épidémiologique de deux cas d'infection par le virus H5N1 dans une même famille au Vietnam a suggéré que l'exposition au cours de la baignade ou de la toilette à l'eau d'un canal potentiellement contaminée pourrait expliquer ces

2 infections (de Jong et al., 2005). Plus récemment, des enquêtes de séroprévalences réalisées au sein de villages cambodgiens où le virus H5N1 avait été confirmé chez les volaille domestique et où des cas humains avaient été déclarés ont identifié l'eau contaminée comme un facteur de risque potentiel (Vong et al., 2008; Vong et al., 2009). Une meilleure connaissance des conditions de survie du virus dans l'environnement permettrait de mettre en place des mesures de lutte ciblées visant à limiter la durée des épidémies et leur résurgence. De telles mesures permettraient de compléter celles déjà prises et qui visent surtout à limiter la transmission directe.

2.2. Le virus H5N1 dans le monde

Le virus influenza A H5N1 demeure une préoccupation mondiale majeure en raison de l'évolution rapide, de la diversité génétique, du large spectre d'hôte, et de la circulation discontinues chez les oiseaux sauvages et domestiques. L'influenza aviaire hautement pathogène A (H5N1) a été à l'origine d'épizooties dans au moins 62 pays en Asie, en Europe, au Proche-Orient et en Afrique et la FAO considère le virus H5N1 à présent endémique chez les volailles dans cinq pays : le Bangladesh, la Chine, l'Égypte, l'Indonésie et le Viet Nam (WHO/FAO/OIE). L'OMS a signalé des cas humains de grippe aviaire A (H5N1) en Asie, en Afrique, le Pacifique, en Europe et au Proche-Orient. Le plus grand nombre de cas humains ont été signalés en Indonésie, au Vietnam et en Egypte, chacun ayant rapporté plus de 100 cas (ces trois pays représentent 79% de tous les cas humains) (WHO, 2011a). Aucun cas humain n'a encore été signalé en Europe occidentale ou en Amérique, bien que le virus H5N1 ait été détecté chez les volailles en Europe (Van Kerkhove et al., 2011).

Les épizooties à IAHP H5N1 ne devraient pas connaitre de ralentissement significatif à court terme.

Tableau 1: Nombre cumulatif de cas humains confirmés d'infection par le virus H5N1 (chiffres rapportés à l'OMS en date du 10 octobre 2011)

Graphique 1: Epidémies de grippe aviaire H5N1 HP chez les volailles (chiffres allant de la fin de l'année 2003 jusqu'au 13 octobre 2011; source WHO)

Table 1: Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011

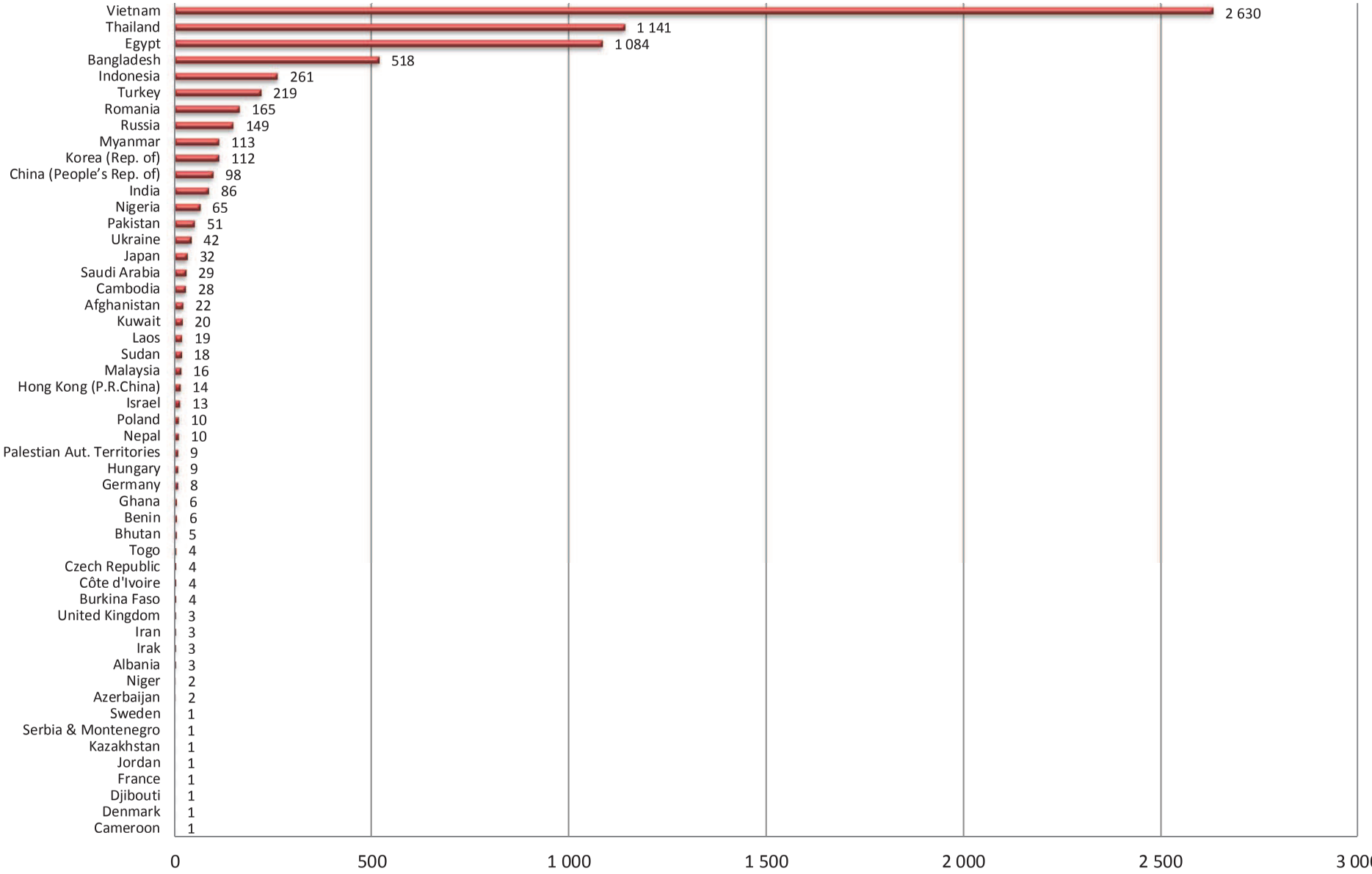
Country	2003		2004		2005		2006		2007		2008		2009		2010		2011		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	3	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	18	16
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	0	0	40	26
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	32	12	151	52
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	8	6	179	147
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	0	0	119	59
Total	**4**	**4**	**46**	**32**	**98**	**43**	**115**	**79**	**88**	**59**	**45**	**33**	**73**	**32**	**48**	**24**	**50**	**26**	**566**	**332**

Total number of cases includes number of deaths
WHO reports only laboratory cases
All dates refer to onset of illness

Source: WHO/GIP, data in HQ as of 10 October 2011

World Health Organization

Outbreaks of Highly Pathogenic Avian Influenza (subtype H5N1) in poultry*
From the end of 2003 to 13 October 2011



*** The addition into the graph of outbreaks of HPAI H5N1 reported from Egypt and Indonesia has been suspended since 26/09/2006 for Indonesia and 07/07/2008 for Egypt, dates for which they declared the disease as endemic. The number of outbreaks of HPAI for these two countries could be found in their respective six-monthly reports.**

2.3. Le virus H5N1 en Asie du sud-est

En 1997, le premier foyer de HPAI à H5N1 a été détecté dans un élevage de poulets à Hong Kong (Shortridge et al., 1998). Malgré les mesures de contrôle drastiques mises en place, de nouveaux cas sont réapparus en 2002, toujours à Hong Kong, mais cette fois également chez des oiseaux aquatiques sauvages dans des parcs naturels (Guan et al., 2002). Des cas de grippe aviaire H5N1 ont ensuite été observés en Chine en décembre 2003 chez des volailles (Li et al., 2004). Dès janvier 2004, on retrouvait le virus dans huit pays voisins : Cambodge, Chine, Corée du Sud, Indonésie, Japon, Laos, Thaïlande et Vietnam, et en août 2004 la Malaisie était également touchée (WHO, 2011b).

Cette propagation fulgurante s'expliquerait par les liens commerciaux ainsi que par les conditions d'élevage particulières qui existent dans ces pays. En effet, beaucoup d'élevages avicoles autorisent une grande proximité entre oiseaux sauvages et domestiques (Gilbert et al., 2008). A partir de juillet 2005, l'épizootie se transforme en panzootie puisqu'elle s'étend vers l'ouest (Kazakhstan, Russie, région des Balkans, Turquie, Croatie) et atteint l'Europe de l'Ouest et l'Afrique en 2006 (WHO, 2011b). La majorité des cas déclarés en Europe ont été observés chez des oiseaux sauvages, et plus rarement chez des espèces domestiques, alors que l'inverse était observé en Asie (Poireau and Dufour, 2007). L'expansion mondiale de la maladie est probablement due à une combinaison de facteurs, mais on peut principalement mettre en cause le commerce légal ou illégal de volailles et de leurs produits dérivés, ainsi que les migrations d'oiseaux sauvages (Dufour, 2008).

Depuis sa détection en 1997, l'influenza aviaire hautement pathogène H5N1 a considérablement affecté la santé humaine et les économies des pays d'Asie du sud-est. Près de la moitié des pays d'Asie ont déclaré des infections H5N1 chez les humains. Celles-ci représentent 82% du nombre total de cas confirmés, et 90% des décès dans le monde entier (Kruy et al., 2008; WHO, 2011a).

2.4. Le virus H5N1 au Cambodge

Le Cambodge a été menacé par des réémergences périodiques du virus H5N1. Dans ce pays, on recense 28 foyers épizootiques déclarés depuis 2004 (Graphique 1). Ces chiffres sont tirés des déclarations à l'OIE, et on peut raisonnablement penser que nombre d'épidémies soient passées inaperçues, et ainsi n'aient pas été déclarées. En effet, le système de déclaration obligatoire dans des pays en développement tels que le Cambodge est très difficile à maîtriser et à gérer efficacement en raison du manque d'information et de moyens de communication dans les zones rurales.

Les animaux concernés sont aussi bien des poulets que des canards, le plus souvent élevés en liberté. Les mares, les rizières sembleraient jouer un certain rôle dans la persistance du virus dont les canards seraient le réservoir principal. Des prélèvements de terre, de sol, de boue, d'eau, etc..., prélevés dans une ferme dont les volailles avaient été touchées par une épidémie de virus H5N1 ont été analysés et ont permis de mettre en évidence la présence d'ARN du virus (Vong et al., 2008). Le travail de Vong, Cavailler et collaborateurs a aussi montré que l'eau est le principal facteur de risque de contamination asymptomatique d'individus à la suite de baignage dans les mares et dans les points d'eau des villages (Cavailler et al., 2010; Vong et al., 2009).

Les premiers cas humains cambodgiens ont été détectés en 2005 et ont été les premiers d'une série de 18 cas (dont 16 mortels) s'échelonnant entre 2005 et 2011 (tableau 1). Les contaminations humaines sont favorisées au Cambodge et dans les pays voisins par le mode d'élevage traditionnel dans lequel on observe une libre circulation des volailles au sein du village ainsi qu'une proximité et des contacts étroits avec les hommes, notamment lors des abattages (Buchy, 2008; Buchy et al., 2007; Vong et al., 2009). À la différence de son voisin vietnamien, le Cambodge n'a pas eu recours à la vaccination de masse des élevages aviaires contre l'hémagglutinine H5, intervention coûteuse, exigeant une organisation très rigoureuse et la mise en oeuvre de mesures lourdes et contraignantes (Kruy et al., 2008).

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Review

A(H5N1) Virus Evolution in South East Asia

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Abstract: Highly Pathogenic Avian Influenza (HPAI) H5N1 virus is an ongoing public health and socio-economic challenge, particularly in South East Asia. H5N1 is now endemic in poultry in many countries, and represents a major pandemic threat. Here, we describe the evolution of H5N1 virus in South East Asia, the reassortment events leading to high genetic diversity in the region, and factors responsible for virus spread. The virus has evolved with genetic variations affecting virulence, drug-resistance, and adaptation to new host species. The constant surveillance of these changes is of primary importance in the global efforts of the scientific community.

Keywords: avian influenza; H5N1 virus; evolution; South East Asia

Introduction

The Highly Pathogenic Avian Influenza (HPAI) H5N1 virus has dramatically affected human health and economies throughout South East Asia since its detection in 1997. Nearly half of the countries in Asia have declared H5N1 infections in humans. These account for 82% of the total number of confirmed cases, and 90% of fatalities worldwide [1,2]. The first H5N1 outbreak in poultry occurred in China in 1996 but the first human case was detected in Hong Kong in 1997 [3]. A large wave of HPAI

H5N1 infections emerged in East and South East Asia in December 2003, rapidly affecting seven countries (Cambodia, China, Indonesia, Japan, Lao People's Democratic Republic (Lao PDR), Thailand and Vietnam) [3-5] . The virus is now endemic in poultry in many of these countries and has caused repeated zoonotic infections in humans [2,6-8]. The virus has implications far beyond its impact on public health. H5N1 viruses have caused widespread disruption to poultry production and trade, particularly in South East Asia where a significant proportion of the population depends on farming for livelihood.

Like all influenza viruses, the HPAI H5N1 virus is able to rapidly evolve via mutations and reassortments of its segmented RNA genome. The prototype virus A/Goose/Guangdong/1/96 (A/Gs/Gd/1/96), which emerged in China in 1996, has undergone various genetic reassortments, and spread to neighboring countries in 2003. The direct precursor of the A/Gs/Gd/1/96 virus is unknown but is thought to be a Low Pathogenic Avian Influenza (LPAI) virus circulating in wild aquatic birds [9]. The probable progenitors that have contributed genetic elements to the A/Gs/Gd/1/96 lineage are probably H3N8 and H7N1 viruses from Nanchang (China), and H1N1 and H5N3 viruses from Hokkaido (Japan) [10].

In this review we examine the evolution of H5N1 viruses in South East Asia, the spatial and temporal transmission of H5N1 within South East Asia, and the emergence of clades and genotypes at a country level. Finally, we discuss significant genetic drift and shift mutations emerging in the H5N1 virus in South East Asia, and their significance in terms of drug-resistance, adaptation to different hosts' species, and pandemic threat.

History of emergence and circulation of H5N1 virus in South East Asian countries

China

The Gs/Gd virus was initially isolated from geese in Guangdong Province, China in 1996. The Gs/Gd lineages are capable of zoonotic infections of human hosts via direct contact with infected birds. In Hong Kong SAR in 1997, a derivative of this strain caused the first fatal human case of H5N1 infection [11,12]. Southern China has often been proposed to be an epicenter for the generation of influenza pandemics, and indeed this region has continuously demonstrated the greatest H5N1 genetic diversity, suggesting that in addition of being the country of origin of H5N1 HPAI strains, Southern China is also an ongoing source of emergent and re-emergent HPAI viruses [13].

The circulation of over 20 different HPAI H5 and H7 subtypes has been documented in the last 100 years, however, only the Gs/Gd lineage has become so geographically widespread. Since 2000, HPAI H5N1 viruses have been repeatedly detected in Hong Kong SAR in live poultry markets and multiple novel genotypes have arisen through reassortment of Gs/Gd lineage viruses [4,14,15]. The analysis of 318 viruses isolated from 1996-2006 revealed that Gs/Gd lineage generated a total of 44 different reassortants in China [16].

The Hong Kong outbreak of 1997 was caused by a Hong Kong/156/97–like virus that derived its HA gene from Gs/Gd-like viruses and other genes from H9N2 or H6N1 viruses found in quail and other game birds sold in live markets [17,18]. During this outbreak, 18 humans were infected with H5N1 [2]. Six of these cases were fatal, giving a fatality rate of only 33%, which was much lower than

subsequent H5N1 fatality rates. In response to the Hong Kong outbreak in 1997, millions of poultry livestock were culled, and as a result, a virus with the same gene constellation has not been detected again, indicating that the control measures used during this outbreak resulted in the eradication of this virus lineage.

H5N1 outbreaks re-emerged in poultry in 2003, and continued to spread throughout China in 2004, prompting a mass culling campaign to eradicate the virus. A total of 98 outbreaks in poultry were reported to the World Organization for Animal Health (or Office International des Epizooties, OIE) between 2003 and 2009 [19]. H5N1 has also been detected in wild birds in 2002 and during each winter season from 2004 to 2009 [5,20,21]. Outbreaks in poultry have again occurred in late 2008/early 2009 [5].

The H5N1 virus was not detected in humans again until February 2003 when two cases were reported in Hong Kong in a family who had recently travelled to Fujian province. Additionally, in November 2003, a human case was identified in Beijing [2]. Since late 2005, human cases have been reported from time to time in several Chinese provinces, and to date a total of 38 human cases have been confirmed in China, including 25 fatalities [2].

In 2005, hundreds of wild migratory birds became infected with H5N1, causing mass die-offs in Qinghai Lake in central China [22-25]. Since 2005, only two other outbreaks have been reported in Qinghai province [5]. In April 2006, the H5N1 virus was detected in wild birds, but did not significantly spread. Interestingly, the latest outbreak was reported on the 17th May, 2009 in Qinghai province, at Genggahu Lake. In this outbreak, 121 wild birds were found dead, and 600 backyard poultry were subsequently culled.

Vietnam

H5N1 virus was introduced to Vietnam in late 2003 from Yunnan province, China, causing multiple poultry outbreaks and finally establishing itself endemically in this population [4,8,26] Outbreaks in poultry were first reported in 2004 and shortly followed by several human cases. Infections in humans were continuously reported throughout 2004-2005, with 65 cases reported during this period which at the time far outnumbered any other country [3]. Vaccination of poultry against H5 virus was initiated in Vietnam in August 2005, with inactivated H5N1 and H5N2 vaccines [27]. In efforts to control or eradicate H5N1 in poultry and to prevent human exposure, Vietnam has strengthened their vaccination campaign and associated surveillance programs in poultry, and improved poultry import controls and virological surveillance. Following the vaccination campaign, no influenza outbreaks were reported in poultry or humans from December 2005 until August 2006 [3]. The virus reappeared in poultry but no human cases were reported again until June 2007 and subsequently new human cases were regularly reported until the current time. At the time of writing, the total number of human infections detected in Vietnam was 111 with 56 mortalities, representing nearly one third of worldwide laboratory-confirmed human H5N1 infections [3,28]. Among 50 countries reporting H5N1 avian influenza in domestic poultry to the OIE, Vietnam has reported the greatest number, with 2539 outbreaks declared [5].

Thailand

Avian influenza H5N1 virus was first reported in Thailand in January 2004 [29]. Several outbreaks were reported during the 2004-2005 period across many Thai provinces [29,30], prompting the government of Thailand to implement strict control and prevention measures against HPAI. The primary objectives were to identify geographic areas with confirmed H5N1 disease in poultry (e.g. 5-kilometer radius around an infected flock); to establish controls on the transport of poultry and poultry products out of affected areas, and to promote safe food-handling practices [29,31,32]. Small epidemics were reported in backyard flocks in 2006. However, following the tighter controls introduced during the same year, outbreak numbers and sizes were greatly reduced, leading to a much smaller natural reservoir which persists through the dry months [33]. Sporadic outbreaks continued to occur until the beginning of 2008 [34]. H5N1 transmission is seasonal in Thailand, as there have been several reports of backyard chicken deaths during the wet and cooler months of 2006/07 and 2007/08 [5,29,30,32-34].

Thailand has experienced 1141 outbreaks of H5N1 avian influenza in domestic poultry between 2003 and 2009 [19]. Approximately 63 million birds in three outbreaks were culled to prevent the further spread of the infection [30]. To date, 25 laboratory-confirmed human cases have been reported, including 17 fatalities [3]. The last human case of H5N1 was confirmed in September 2006 in a North-Eastern Thai province [3].

Cambodia

In Cambodia the first confirmed outbreak of HPAI subtype H5N1 in poultry was reported during January 2004, and was followed by 14 other H5N1 outbreaks during the same year [35]. Interestingly, a retrospective investigation also revealed that between December 2003 through January 2004, a HPAI H5N1 outbreak occurred in a wildlife center in Takeo province, infecting several captive wild birds species, and cats. During the first four months of 2005, four fatal human H5N1 cases were detected in Kampot province, South East Cambodia, coinciding with deaths among poultry in this province [3]. In 2006, four outbreaks were detected in domestic poultry, and two human cases were reported [36,37]. In April 2007, Cambodia confirmed the seventh fatal human case of H5N1 infection from Kampong Cham province. The most recent case of human H5N1 infection (8th human case) was also the first non-fatal case and occurred in December 2008 in Kandal province. From 2004 to date, Cambodia officially reported 21 outbreaks of H5N1 avian influenza in domestic poultry [19]. The control strategies in poultry are based exclusively on culling of infected flocks, and the prohibition of poultry imports [35].

Lao PDR

H5N1 avian influenza was detected in poultry in Lao PDR in 2003. A mass culling campaign was rapidly conducted, resulting in the loss of approximately 155,000 poultry. Surveillance of live bird markets during 2005 and 2006 failed to detect the virus, or serological evidence of exposure to the virus, suggesting that the virus from the initial outbreak had been eradicated [38]. In February 2006,

avian influenza H5N1 virus was isolated from healthy ducks at a farm in Vientiane. Two human cases (both fatal) were reported in 2007 from Vientiane Province [2] In 2008 and early 2009, Lao PDR reported new poultry outbreaks of H5N1, however, no human cases were declared [3]. Since 2003, a total of 18 H5N1 outbreaks in poultry have been reported by Lao PDR authorities [19].

Myanmar

Myanmar reported its first outbreak of H5N1 virus in poultry in March 2006, and subsequent outbreaks were reported in February, October, November, and December 2007 [3]. The sole human case of H5N1 infection (non-fatal) was reported in December 2007, in the Shan State province where an outbreak in domestic birds occurred at the same time. In March 2008, H5N1 sero-positive ducks were detected during routine surveillance conducted in the Shan State province [3]. To date, the country reported a total of 93 H5N1 avian influenza outbreaks in domestic poultry [19].

Indonesia

Indonesia has suffered the heaviest burden of avian influenza, with 141 human infections, of which 115 were fatal [2]. Indonesia first detected H5N1 outbreaks in poultry in December 2003, and then continuously through to August 2006. H5N1 virus is now endemic in many Indonesian islands, such as Java, Sumatra and Sulawesi. To date, 261 outbreaks in poultry were reported in the country [19]. The first human case was reported in July 2005, and then human infections continued to occur frequently until the current time. Three family clusters of H5N1 infection were identified in 2005 [39]. In May 2006, Indonesia reported a large family cluster involving 7 family members and human-to-human transmission was suspected [40].

Malaysia

Malaysia reported outbreaks its first outbreaks in poultry in August and September 2004. Additional outbreaks were reported in February and March 2006 in free-range poultry flocks and then again in chickens in June 2007. No human cases were detected in Malaysia [41]. Early detection and drastic culling measures could be attributed with such a successful control of H5N1 outbreaks in this country. A total of 16 H5N1 outbreaks in poultry were reported by Malaysian authorities [19].

Comparison of fatality rates in patients from South East Asian countries

The 1997 H5N1 outbreak in humans in Hong Kong resulted in a fatality rate of 33%. Since the re-emergence of H5N1 in 2003, this rate has been considerably increasing in most affected countries [2]. Of 429 confirmed human cases worldwide, 61% were fatal. South East Asia recorded 326 of these cases, including 222 fatalities, giving a fatality rate of 68% [2,42]. This rate is variable within each country. For example, China's overall fatality rate is 66%, Indonesia's 82%, Thailand's 68%, Vietnam's 50% (Table 1). The high fatality rate in Indonesia is striking, given the significant number of cases that have been reported there. Other South East Asian countries, such as Cambodia, Lao PDR,

Myanmar, have small numbers of confirmed human cases (8, 2, 1, respectively), which means interpreting their fatality rates (88%, 100%, 0%, respectively) is difficult.

It is unclear whether H5N1 viruses in certain geographical regions differ in their pathogenicity. It would seem that clade 2.1 viruses (Indonesia) are more pathogenic than clade 1 viruses (Cambodia/Thailand/Vietnam) and 2.3 viruses (China) [43]. Drawing a link between H5N1 clade circulation and fatality rates is difficult, due to differences in health care practices and duration between onset of illness and treatment. Affordability of healthcare in developing countries and reduced access to anti-viral treatment at an early stage of the disease may partially explain increased fatality rates. Where surveillance is lacking, there is the possibility for under-representation of fatality rates, however, subclinical infections may also be under-estimated. Indeed, out of more than 600 blood-tested Cambodian villagers from areas where two children died in 2006, seven were seropositive for H5 antibodies indicative of asymptomatic infection. Thus, in this scenario the fatality rate was much lower than originally determined [44].

Table 1. Summary of H5N1 outbreaks in poultry and humans in South East Asia.

Country	Year of first outbreak	Number of outbreaks in poultry	Number of human infections	Number of human deaths	Country Fatality Rate
Cambodia	2003	21	8	7	88%
China	1996	98	38	25	66%
Indonesia	2003	261	141	115	82%
Lao PDR	2003	18	2	2	100%
Malaysia	2004	16	0	0	-
Myanmar	2006	93	1	0	0%
Thailand	2004	1141	25	17	68%
Vietnam	2003	2539	111	56	50%

Evolution of the Predominant Genotypes in South East Asia

South East Asian HPAI H5N1 viruses have their HA and NA genes derived from the prototype A/Gs/Gd/1/96 virus, whereas the genes encoding the six internal proteins (PB2, PB1, PA, NP, M, NS) are derived from several other sources. This diversity has allowed reassortment into various genotype groups, defined as unique gene constellations [4,14,16]. As for each of the internal gene segments constituting these constellations, a Neighbour-Joining bootstrap support above 70% or Bayesian posterior probability above 95% are the determinants for a distinct phylogenetic lineage [16].

However, in some cases, the same unique gene constellation has led to the definition of two genotypes, which only differ by some molecular marker. Genotypes Z and Z+, for example, differ by the presence or absence of a multi-amino-acid deletion on the NA protein [16].

By 2001, eight genotypes (A, B, C, D, E, X_0, W, Z) had been identified in Hong-Kong and China (Figure 1), derived from A/Gs/Gd/1/96-like viruses, from an H9N2 avian virus (Chicken/Hong Kong/Y280/97), and other unknown viruses [4,14,16]. By 2003, three other major genotypes had arisen (Y, Z+, V) [4,16]. In 2004, the reassortment between genotypes Z and W led to the emergence of a new genotype, G [45]. Amongst all these genotypes, only a few persisted for longer than two years (B, Z, Z+, V, G, W, and X_0) suggesting the acquisition of efficient survival capacities (Figure 1) [16].

These multiple reassortment events are believed to have occurred within domestic duck species, in which H5N1 generally causes an asymptomatic infection. It is proposed that avian influenza viruses from the natural gene pool existing in wild birds are introduced into domestic ducks, and are then able to reassort with endemic H5N1 viruses [45]. This would then enable the transmission of newly emerged reassortants to other poultry species, and facilitate their rapid spreading. Until recently, there was no evidence of reassortment events outside of China [16]. However, in 2008 and 2009, studies provided evidence of the emergence of local reassortants in Vietnam, Indonesia, and Thailand [28,33,45,46].

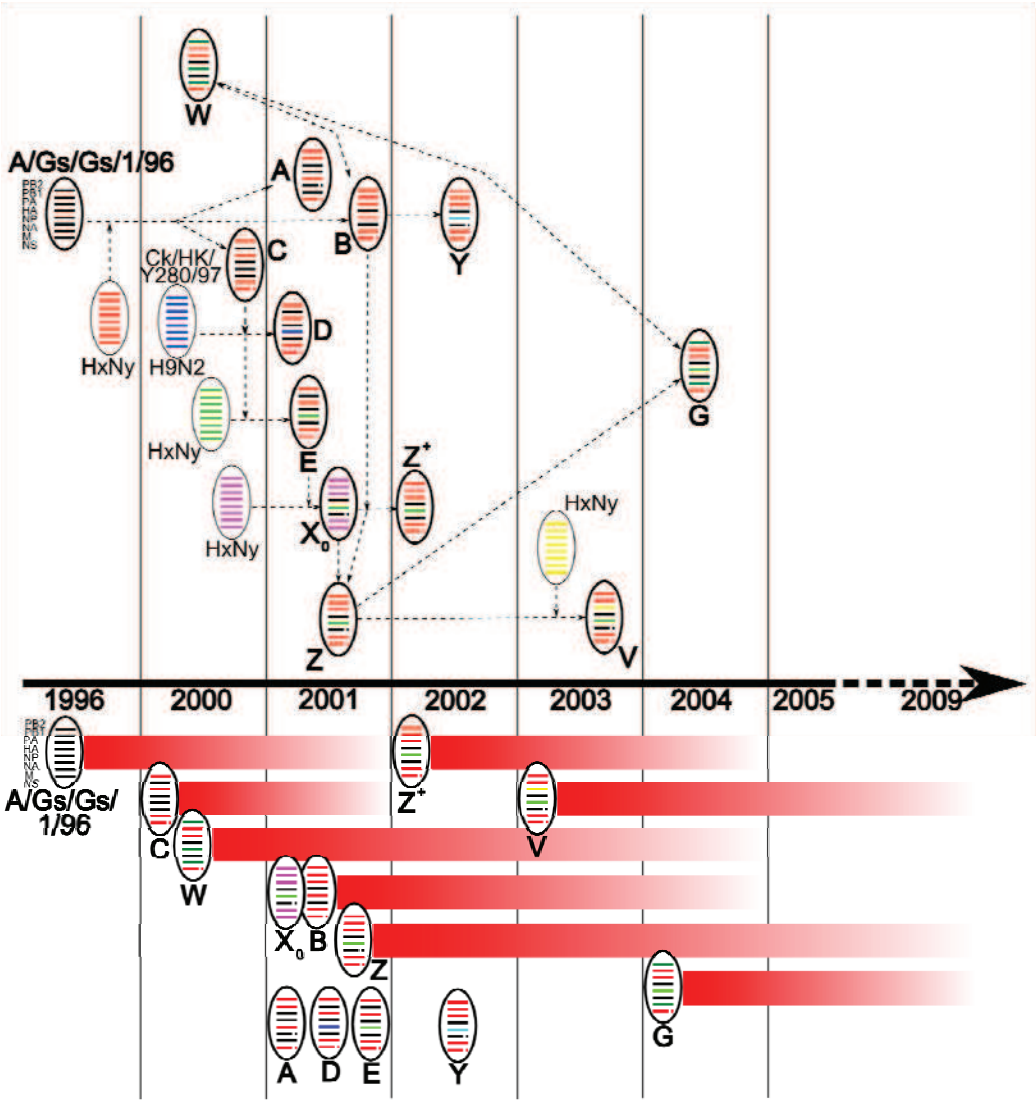
Genotype Z has dominated the H5N1 outbreaks documented in South East Asia from 2003 until the present day [47]. Nevertheless, in 2005 and 2006 several genotype G viruses were detected in poultry in Northern Vietnam, for example A/Duck/Vietnam/568/2005 and A/MuscovyDuck/ Vietnam/1455/2006 [48] (Figure 2). These viruses may have been introduced into Vietnam in early 2005, since they were closely related to a virus first detected in Guangxi, China in that year [47]. Although seven clades and nine genotypes have been identified in Vietnam since first detection of H5N1 virus in the country [28,49], phylogenetic analysis of the internal genes reveal that at least 8 potential ancestors were necessary for the emergence of the viruses that circulated in this country, and subsequently provided a large pool of genes for further reassortments [28].

Although in Indonesia genotype Z is the sole genotype detected, there is now evidence of reassortment events within genotype Z viruses [46]. From 2003 to 2004, all detected viruses belong to a single phylogenetic group [46]. However, from 2005 to 2007, three groups were identified (named 1, 2, 3). Within group 2, subgroups of reassortant viruses were identified which were likely to have resulted from reassortment between group 3 ancestral viruses (M and PB1 genes), and group 2 ancestral viruses (PB2, PA, HA, NP, NA, NS genes). The common ancestor of these reassortant viruses has been dated to July 2005 [46].

All Thai strains of HPAI H5N1 viruses belong to genotype Z or Z^+ with one exception which was a virus isolated in Nakhon Pathom province in 2006, A/Chicken/Thailand/NP-172/2006, which belongs to genotype V [50]. Although viruses from different clades have circulated in Thailand, no reassortment has been reported between clade 1 and 2 viruses. Nevertheless, important reassortment events occurred between various strains of H5N1 virus which persisted silently over a dry season period before giving rise to outbreaks in 2007-2008 [33].

In Lao PDR, the first H5N1 isolates detected in 2003 were genotype Z viruses. However, in 2007, a genotype V strain was isolated, and interestingly clustered with the genotype V A/Chicken/Thailand/NP-172/2006 virus, suggesting a common origin [51]. Suprisingly, only genotype Z clade 1 viruses have been detected in Cambodia since the emergence of the epidemic in 2003 [52].

Figure 1. Diagram representing the emergence and persistence of major H5N1 reassortant viruses. Gene segments are ordered PB2, PB1, PA, HA, NP, NA, M, NS from top to bottom within the virus particle diagram. NS1 deletion (position 80-84) and NA-stalk deletion (position 49-68) are represented by discontinuous gene segments. Virus particles outlined in simple black represent potential donor viruses and those outlined in bold black represent characterized H5N1 genotypes placed at the year of first detection. Particles named "HxNy" represent potential donor viruses of which HA and NA subtypes were not identified. Arrows in dotted lines represent possible reassortment pathways of genotype development. Persistence of each genotype since first year of detection is represented by red lines, under the timeline.



H5N1 Clade Evolution in South East Asia

Ten different clades (genetic groups) have been defined within the H5N1 viruses, based upon the evolution of the H5 hemagglutinin gene. These clades are strictly defined by phylogenetic criteria, such as sharing a common node, reaching a threshold value of ≥ 60 for the bootstrap at this clade-defining node and fitting in a precise range of values for the average percentage pairwise nucleotide distances between and within clades of >1.5% and <1.5%, respectively [53]. Several sublineages have emerged within the different clades, resulting in the designation of subclades.

Between 2003 and 2006, only clades 1 and 2 viruses circulated in South East Asia (Figure 2). Clade 1 viruses have circulated in Thailand, Vietnam, Malaysia, Lao PDR and Cambodia, affecting both poultry and humans. Clade 2 viruses in South East Asia are represented by subclades 2.1 (2.1.1, 2.1.2, 2.1.3) and 2.3 (2.3.2, 2.3.4). Clade 2 viruses have been circulating in Indonesia since 2003. Within Indonesian clade 2 viruses, subclade 2.1.1 viruses have caused outbreaks exclusively in poultry whereas subclades 2.1.2 and 2.1.3 have affected both birds and humans. Clade 2 (subclade 2.3.2) viruses emerged in Vietnam in 2005, infecting poultry and other migratory species. Subclade 2.3.4 viruses then emerged in 2006 and were identified in poultry outbreaks in Lao PDR, Vietnam (mainly in the north), Thailand, and Malaysia but never in Cambodia (Figure 2) [28,54,55]. These viruses were confirmed in human hosts in Vietnam for the first time in 2007 [56].

In Vietnam, seven clades and/or subclades have been identified: clades 0, 1, 2 (2.3.2, 2.3.4), 3, 5 [28], and recently clade 7 [49]. Viruses from clades 0, 3 and 5 persisted no longer than one to two years each [28]. Clade 7 viruses were detected in Northern Vietnam in 2008 (e.g., A/Chicken/Vietnam/NCVD-03/2008) (Figure 2). Subclade 2.3.2 viruses isolated in Vietnam are represented on the HA tree by the strains A/Duck/Vietnam/568/2005 and A/Muscovy Duck/Vietnam/1455/2006 (Figure 2), which are also the representatives for genotype G viruses (Figure 1). It is of interest to note that some viruses, such as A/Duck/Vietnam/37/2007, belong to the subclade 2.3.4, whilst sharing the NA and internal gene constellation of clade 1 viruses, providing evidence for mechanisms of reassortment between different clades (Figure 2 and 3) [48].

Figure 2. Phylogenetic tree of the HA gene of representative South East Asian H5N1 viruses. Analysis of HA gene based on full length gene sequences. The tree was generated by Bayesian analysis using MrBayes v3.1.2 [57]. Numbers on branches indicate Bayesian posterior probability values. The tree was rooted to A/Goose/Guangdong/1/1996. Scale bar: 0.01 substitutions per site. Colors used for the different countries are: Cambodia = light green; Indonesia = dark green; Lao PDR = pink; Malaysia = purple; Myanmar = maroon; Thailand = red; Vietnam = blue.

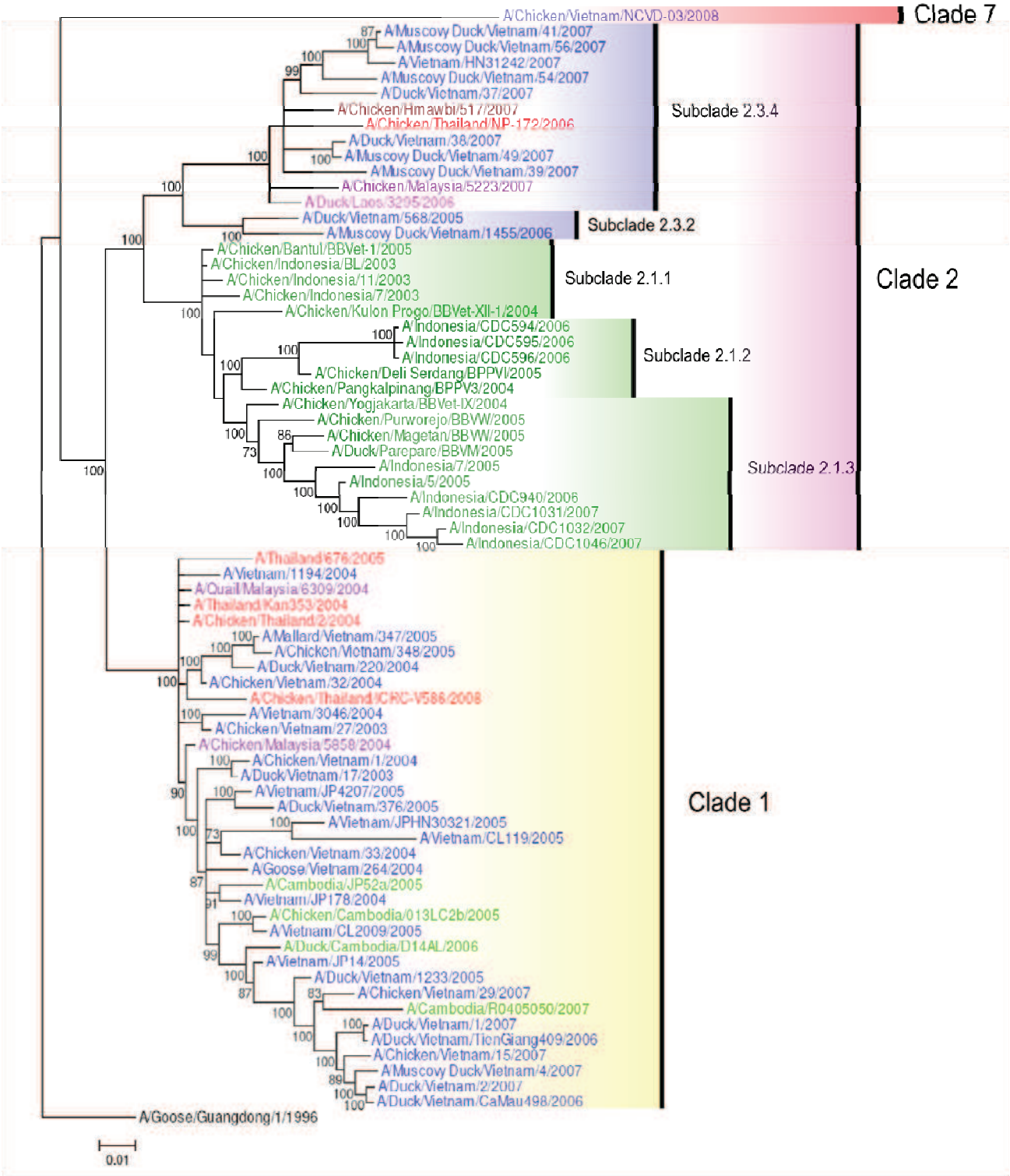
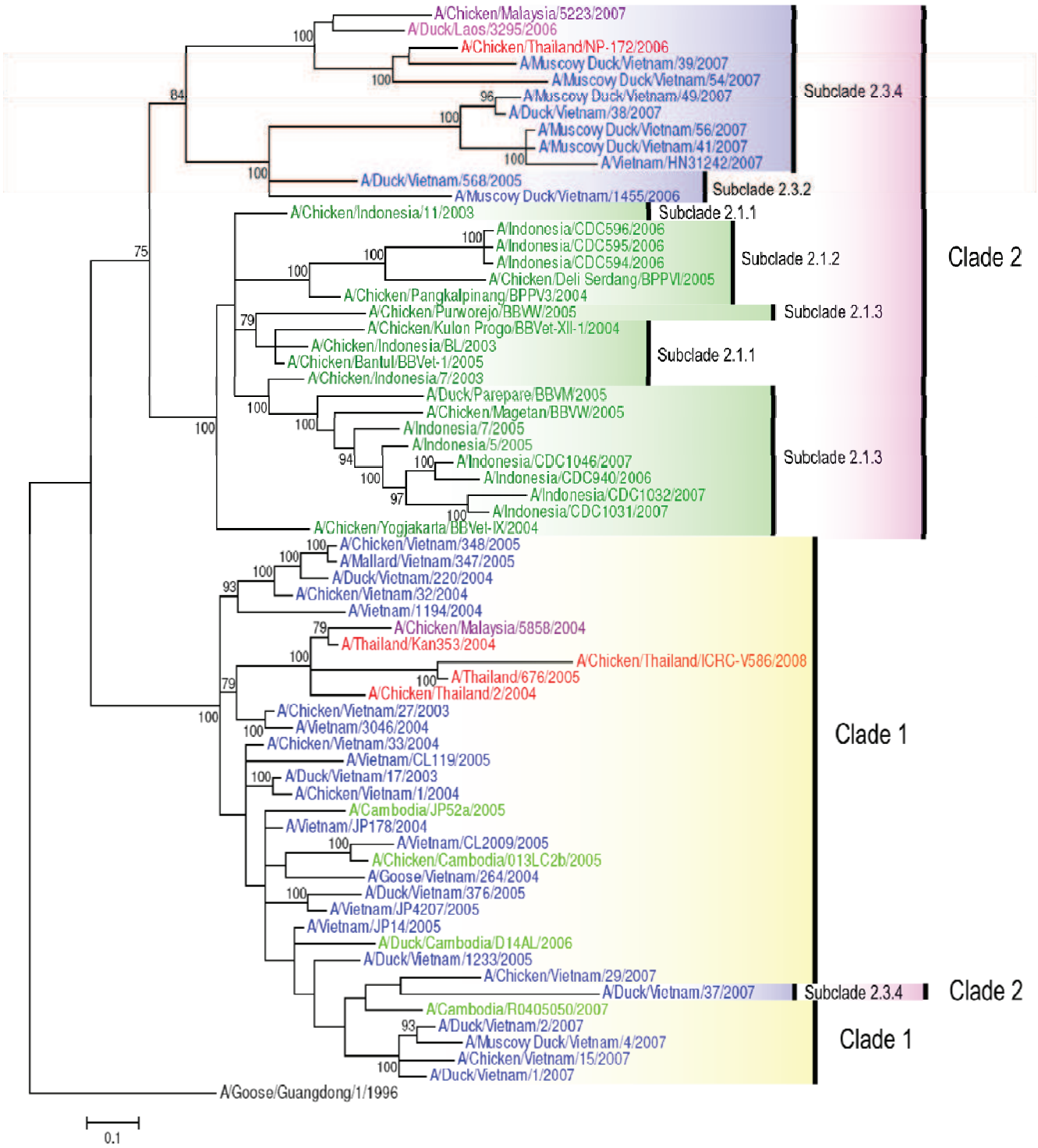


Figure 3. Phylogenetic tree of the NA gene of representative South East Asian H5N1 viruses. Analysis of NA gene based on full length gene sequences. The tree was generated by Bayesian analysis using MrBayes v3.1.2 [57]. Numbers on branches indicate Bayesian posterior probability values. The tree was rooted to A/Goose/Guangdong/1/1996. Scale bar: 0.1 substitutions per site. Colors used for the different countries are: Cambodia = light green; Indonesia = dark green; Lao PDR = pink; Malaysia = purple; Myanmar = maroon; Thailand = red; Vietnam = blue.



Geographical Dynamics of H5N1 Virus Transmission

The H5N1 influenza virus has continued to spread from its established source in Southern China to other regions through the transport of poultry and bird migration [47]. Domestic ducks in Southern China have had a central role in the generation and maintenance of this virus, and wild birds may have contributed to the increasingly wide spread of the virus in South East Asia, and to other parts of the world [4,58,59].

Since 2003, the HPAI H5N1 virus has spread from China to other countries during three successive transmission waves in 2003, 2005, and 2006 [4,60,61]. The first wave of H5N1 outbreaks occurred in 2003/2004. These viruses originated in Yunnan and Hunan and became endemic in South Vietnam and Cambodia (clade 1 viruses), and Indonesia (clade 2.1 viruses), respectively (Figure 2 and Figure 4) [26]. The second wave of transmission occurred following the outbreak at Qinghai lake in Northern China in 2005, whereby clade 2.2 viruses were transmitted by migratory birds to Africa and Europe [22-25]. In the third wave, a Fujian-like sublineage (clade 2.3) replaced previously established sublineages in several provinces of China, before spreading to Vietnam, Thailand, Lao PDR, Malaysia and Myanmar in 2006 [61]. The event currently occurring in Qinghai province may be of some concern, since it is known to be on a major migration route of wild birds and of primary epidemiological importance.

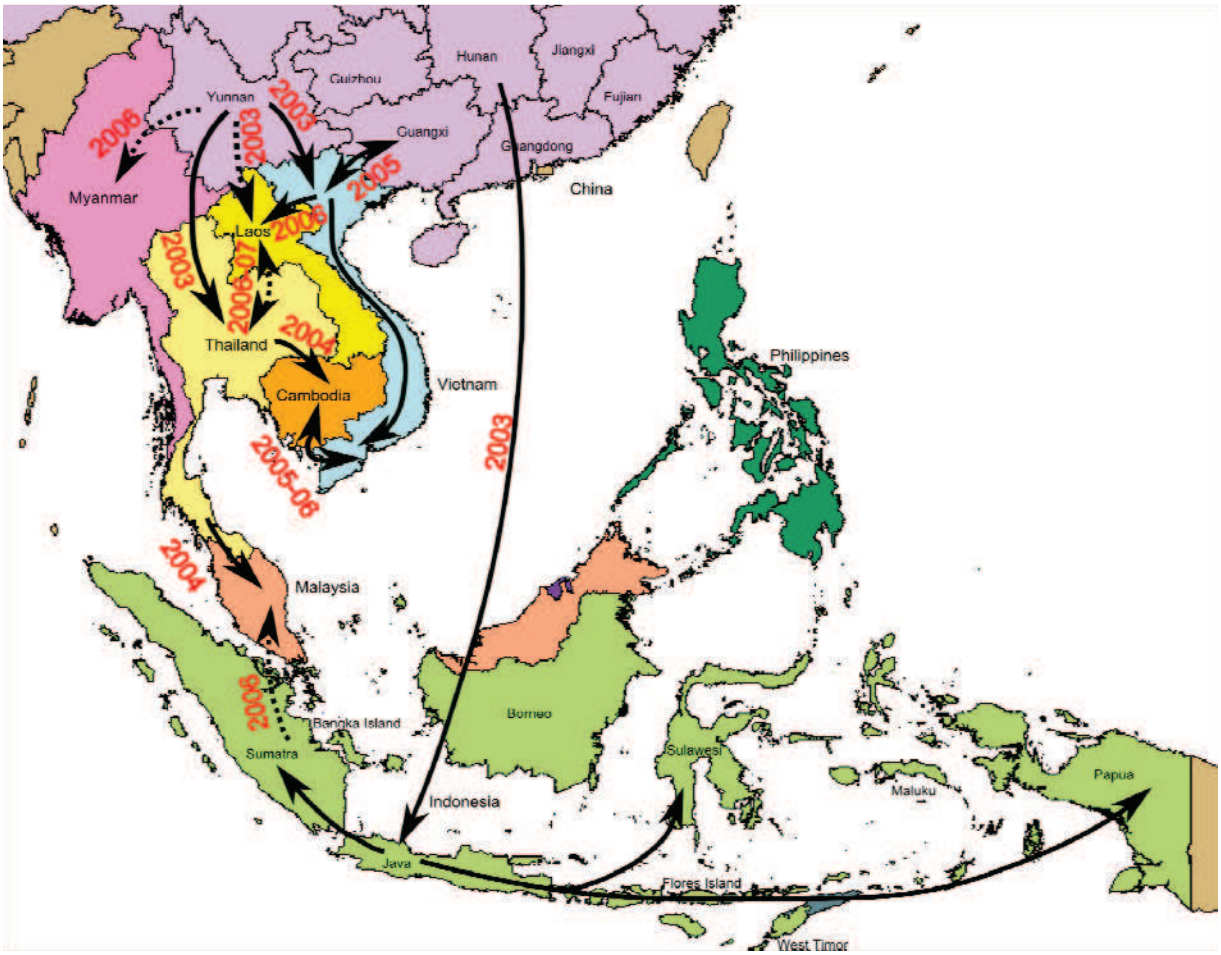
The H5N1 virus has been introduced into Vietnam from Southern China on multiple occasions. The majority of novel viruses were first detected in Northern Vietnam, suggesting introduction from Yunnan (China) (Figure 4). These viruses subsequently spread to Southern Vietnam, often after reassorting with pre-existing local viruses in Northern Vietnam [26,28]. The apparent northern to southern spread of H5N1 may correspond to direct poultry trade routes between major population centres in these regions [58] or to trade routes along the Mekong River from Lao PDR to Vietnam. Some viruses may have spread back and forth between countries at different time points [48]. Cross-border poultry trade between Vietnam and China could have led to the introduction of clade 2.3.4 (presumably from Guangxi province, China) and other lineages into Vietnam [48].

The H5N1 virus became widespread and endemic in Thailand throughout 2004 without interruption, despite brief periods of undetectable transmission during the hottest months of February-May [29]. It is believed that the virus is maintained in Thailand at low level during these dry, hot months, therefore providing a source for outbreaks during the wet and cooler months of September-December, when the conditions are more favourable for its spread. A new viral strain (clade 2.3.4, genotype V) was introduced into the north-eastern region of the country in 2006 [50]. The subtype H5N1 viruses circulating in the markets in ten provinces of central Thailand during July 2006–August 2007 were genetically related to those that circulated in Thailand during 2004–2005, which indicated that the virus was endemic to Thailand [62]. This suggests that yearly re-emerging viruses in central Thailand belonged to a similar lineage and that they originated from a locally persistent reservoir, rather than repeated introductions [30,63]. Recent avian influenza outbreaks in Thailand in 2007-2008 were also shown to be caused by indigenous viruses [33].

In Cambodia, the HPAI H5N1 virus was probably introduced from Thailand in 2004 [64] and then re-introduced from Vietnam over several waves of transmission until the virus finally became

endemic, establishing a recent sub-lineage in the south Indochina peninsula region. Poultry movements, rather than repeated re-introductions of H5N1 viruses by wild birds, are responsible for virus circulation and perpetuation [64]. Within Cambodia, the spread of H5N1 virus clearly occurs in a North-to-South direction, following a major road and transport route. Poultry trading, live poultry markets, cock fights, and other risk factors for poultry contamination are therefore likely to be responsible for the spread of the virus [65,66].

Figure 4. Map of major H5N1 migration events in South East Asia, based on epidemiological evidence. Arrows represent probable transmission routes. Arrows in dotted lines represent migrations for which the exact origin or the direction has not been elucidated.



In Lao PDR, it seems that the H5N1 virus has not persisted endemically. H5N1 was first detected in 2003, and then disappeared in 2004 [38]. In early 2006, a clade 2.3.4 virus was detected, which was presumably introduced from Northern Vietnam [38]. This virus also seems to have vanished from Lao PDR [38]. However, in 2007, a genotype V, clade 2.3.4 strain was isolated near the Thai border. Phylogenetic analysis of the HA gene showed that this strain probably shared a common origin with Thai strains isolated in 2006 near the border. Although the direction of the transmission can not be determined, extensive poultry movement across the Mekong River is believed to have facilitated the circulation of these strains between countries [51].

In Indonesia, phylogenetic analysis suggests that a single introduction of genotype Z, clade 2 viruses from Southern Chinese domestic poultry (Hunan province) has occurred (Figure 4) [4,47,67]. The continuing endemicity of those viruses subsequently resulted in the establishment of geographically distinct groups [8]. In the Indonesian sublineages, there are three groups of viruses. The first group includes viruses from Central and Eastern Indonesia (Java, Southern Sulawesi and West Timor). The second group also contains viruses from Central and Eastern Indonesia (isolates from Java, Bali, Flores Island and West Timor). In comparison, the third group of viruses are from Central and Western Indonesia, found throughout Java and Sumatra, and Bangka Island [8]. These relationships highlight the subsequent spread both east and west throughout the country. Continued virus activities in Indonesia were attributed to transmission via poultry movement within the country rather than through repeated introductions by bird migration [8]. Java is thought to be an epicentre for H5N1 generation and spread in Indonesia [8,68] (Figure 4).

Evolution of Influenza A(H5N1) Virus Genes

Hemagglutinin (HA) gene

Diverse populations of endemic HPAI H5N1 viruses have continuously evolved in South East Asia, from 2003 until the present day, and several gene modifications have occurred within these viruses which may affect their transmissibility and pathogenicity. To become a pandemic strain, a H5N1 virus must be able to be efficiently transmitted between human hosts, a feature that existing H5N1 viruses have not yet acquired.

The HA is the main influenza antigen and determines cell binding, host range, and neutralizing antibody response. Thus, some modifications on the HA gene could provide the virus with advantageous properties. Several key amino acid residues on the HA have been identified.

Avian influenza viruses are defined as HPAI or LPAI viruses, based on the severity of the disease which they cause. HPAI viruses are highly contagious amongst poultry, and often result in a high fatality rate, especially in terrestrial poultry like chicken, quail or turkey (up to 100% in 2-3 days). LPAI viruses are responsible for mild diseases, with few or no symptoms in some bird species. The defining feature of HPAI is a multi-basic amino acid motif at the cleavage site present in the HA gene (PQRERRRKKR/G), which confers increased pathogenicity. LPAI viruses have a single arginine at the HA cleavage site, which can subsequently be cleaved only by trypsine-like proteases. Since these proteases are present in a restricted number of organs, the infection is usually limited to the respiratory or to the intestinal tract, without becoming systemic. In contrast, the presence of multiple basic amino acids allows the hemagglutinin to be cleaved by many different proteases, enabling a broader tissue tropism, and the ability to cause systemic infections. Some H5N1 strains have alterations on this site, such as Arg (R) or Lys (K) deletions or insertions. Alterations of this kind were observed, among others, on Cambodian, Indonesian, Thai and Vietnamese H5N1 isolates [8,49,50]. Although the real impact of such changes in the HA cleavage site is difficult to estimate, the high frequency of these occurrences highlights the importance of conscientious surveillance of this site.

The relevant residues involved in antigenic sites on the H5 HA have been described, and compared to H3 antigenic sites which have been comprehensively characterized [69,70]. The host immune

pressure can induce mutations on these antigenic sites, which results in the emergence of immune-escape mutants. Therefore, a positive selective pressure on those sites can rapidly lead to ineffective host immune responses, which are a major constraint for the development of human or animal vaccines. In Vietnam and Indonesia, among virus isolated from August 2003 to June 2005, a positive selective pressure has been reported on 8 residues of the HA gene. Five of those residues were located on antigenic sites A and E (positions 83, 86, 138, 140 and 141), two of them were suggested to be involved in receptor binding (positions 129 and 175). The last one was a potential *N*-linked glycosylation site (position 156) [8].

The HA of human influenza viruses preferentially bind to sialic acid (SA) receptors linked to galactose (Gal) through an α-2,6 linkage (SA α-2,6Gal), whereas avian influenza viruses preferentially bind to SA receptors of the α-2,3 linkage (SA α-2,3Gal). The HA sites which bind these receptors are often under selective evolutionary pressure. Mutations of these binding sites from an avian to human receptor binding preference could facilitate human-to-human transmission and these sites have been proposed as markers for assessing the pandemic potential of H5N1 isolates [71]. Two key residues have been identified to determine receptor preference switching – position 226 and 228 in H2 and H3 isolates, which corresponds to 222 and 224 in the H5 gene [72]. However, the mutations Gln222Leu and Gly224Ser which are believed to enhance the affinity of the virus for the SA α-2,6Gal have never been observed in H5N1 field isolates. Nevertheless, several naturally occurring mutations were associated with an enhanced affinity of the avian virus to "human type" receptors SA α-2,6Gal. Mutations at position 182 (Asn182Lys) and 192 (Gln192Arg) can independently switch the receptor binding preference from avian to human [73]. Several other combinations of mutations were found to have a variable effect on receptor switching, certainly indicating that in the right circumstance a virus may adapt to bind human receptors more efficiently.

H5N1 HPAI viruses from clade 2.1 (found in Indonesia) may be under a lower positive selective pressure compared to the other clades [74]. Although such an observation is difficult to interpret, there may be a reduced necessity for the virus to evolve and adapt in Indonesia due to the high endemicity of the disease. The persistence in poultry reduces the need for the virus to jump from one species to another, especially to mammalian species, in order to maintain the chain of transmission [74].

Neuraminidase (NA) gene

The neuraminidase enzyme cleaves HA from sialic acids, facilitating the release of newly assembled viral particles from the host cell surface and thus enabling spread to other cells. Interestingly, all H5N1 viruses which have been isolated post-2003 contain a deletion in the stalk region of the protein, at positions 49-68 for clade 1 and 2 viruses, and at positions 54-72 for clade 3 viruses [75]. This deletion reduces the enzymatic activity of the NA, and may act to balance with the HA which has a reduced affinity interaction with receptors in poultry, compared to aquatic birds [76].

The NA is a target for the neuraminidase inhibitor (NAI) class of antiviral drugs, including Oseltamivir (Tamiflu^{TM}) and Zanamivir (Relenza^{TM}) which are commercially available. H5N1 viruses are typically sensitive to NAI's , and these drugs are used as first line treatment for H5N1 infections in humans, and are a major component of pandemic planning as they are also recommended for

chemoprophylaxis [77]. The His274Tyr mutation is associated with resistance to Oseltamivir. This mutation is now increasingly prevalent in seasonal H1N1 and H3N2 influenza strains, partly due to widespread use of Oseltamivir [77]. In addition to His274Tyr, amino acid substitutions Arg292Lys, Glu119Val and Asn294Ser have been associated with resistance or reduced sensitivity to Oseltamivir [78].

There have been several reports illustrating the development of Oseltamivir resistance during the treatment of H5N1-infected patients. Some patients developed the His274Tyr mutation, others the Asn294Ser mutation associated with a decreased sensitivity to NAI's [79,80]. To assess the threat of a resistant H5N1 strain persisting with equal fitness and replicability, the H5N1 virus A/Vietnam/1203/2004 was engineered to possess several NA resistance mutations including His274Tyr, Glu119Val, Arg292Lys, and Asn294Ser. Viruses with His274Tyr and Asn294Ser were found to retain their infectivity and pathogenicity, highlighting the importance of ongoing surveillance of Oseltamivir resistance for early detection of resistant viruses [81].

In the absence of resistance mutations, genetic drift mutations also give rise to variations in Oseltamivir sensitivity [82,83]. Mutations which are remote from the active site may not confer absolute resistance, but may affect the dosage of drugs required to treat these infections. Suboptimal dosing may lead to the emergence of fully resistant viruses. Clade 2.3.4 viruses in Vietnam were recently found to be 8-fold less susceptible to Oseltamivir, but maintain their susceptibility to the adamantanes (M2 inhibitor drugs) [56]. Treatment combining NAI and adamantanes is recommended for patients infected with adamantane-sensitive H5N1 strains [56,84]. Ongoing evaluation of the evolution of drug sensitivity in both seasonal and H5N1 influenza viruses is vital in understanding the best treatment options in the wake of rapid virus evolution.

M gene

The M gene encodes two capsid proteins (M1 and M2). M1 is a RNA-binding protein, and M2 is a membrane ion channel protein involved in H^+ proton transport, which facilitates the acidification of the endosome and the release of the viral particle inside the cytoplasm [85]. A comparison of synonymous and non-synonymous nucleotide substitutions in virus isolates from Indonesia and Vietnam (2003-2005) provided evidence of positive natural selection on the M2 gene [8]. Mutations on the M2 protein can result in different adaptative processes which affect the virus' capacity to uncoat during the early stages of the infection, and modulate its virulence. Also, mutation on the M2 gene can lead to adaptation to the different pH environments of aquatic or terrestrial hosts [8]. Moreover, the M2 protein is the target of the adamantane class of antiviral drugs (amantadine, rimantadine). Two mutations on this protein (Leu26Ile and Ser31Asn) lead to drug resistance, and these mutations are present in all genotype Z, clade 1 viruses [86]. Clade 2.3.4 viruses from Indonesia and Vietnam remain sensitive to amantadine [56], and clade 2.1 and 2.2 viruses have varying rates of resistance to this class of drug [86,87]. Thus, monitoring M2 mutations in H5N1 viruses remains an important aspect of avian influenza surveillance.

Polymerase complex (PB1, PB2, PA)

The polymerase complex of influenza viruses is composed of three subunits: PB1, PB2, and PA. These subunits, along with the NP protein and the RNA genome, form the ribo-nucleoproteic complex, necessary for the protection of the genome, as well as the replication and transcription processes. The PB1 gene also encodes the PB1-F2 protein by an alternative reading frame. Analyses conducted on viruses from Vietnam and Indonesia isolated between 2003 to 2005 showed that several sites on the PB1-F2 gene were under positive natural selection, although the significance of these particular sites is not known [8]. In mouse models and in cell lines, the PB1-F2 protein can influence viral pathogenicity, by sensitizing host cells to apoptotic stimuli (e.g. TNFα), thereby promoting apoptosis during infection [88,89]. However, the residues involved in this process have not yet been identified.

Mutations at residue 627 on the PB2 protein are associated with increased virulence and are thought to be relevant to the adaptation of H5N1 viruses to human hosts. The 627 residue on PB2 is a glutamic acid (Glu) in avian strains [76]. The Glu627Lys mutation on the PB2 improves replication efficiency, enhances adaptation of HPAI viruses to mammalian hosts, and increases transmission between mammalian hosts in experimental models [90-92]. The 627Lys mutation enhances growth at lower temperatures, consistent with those found in the human upper respiratory tract (around 33°C), compared to wild type viruses with 627Glu which grow optimally at 41°C [93,94]. Within clade 1 or clade 2.1 viruses, the Glu627Lys mutation is observed in some human strains but not in avian strains, suggesting a selective advantage for these isolates in mammalian hosts [8,95]. In clade 2.2 viruses, the Glu627Lys mutation is observed in both human and avian strains [47]. Interestingly, most of the strains isolated in birds from the Qinghai Lake outbreaks in 2005 (clade 2.2), which spread to Europe and Africa, contained the 627Lys mutation [60,96].

In addition to residue 627, several other changes on the PB2 protein and other proteins of the polymerase complex also contribute to adaptation to mammalian hosts and to regulation of virulence [90,97]. Positive selective pressure was also detected at several sites of unknown function, on PB2 and PA genes from Indonesian strains isolated from 2003 to 2007 [39]. Thus, surveillance of the substitutions occurring on the polymerase genes, and further elucidation of the importance of PB2 and PA genes in virus evolution is of ongoing interest.

NS1 protein

The non-structural protein NS1 can regulate H5N1 virus virulence in humans by modulating the host immune response. The severity of human infections with H5N1 virus in 1997 was partly due to the resistance of these viruses to interferons and TNFα during the host immune response. The presence of glutamic acid at residue 92 on the NS1 was vital for these effects [98,99]. However 92Glu has not been identified in more recent human H5N1 strains. Ser42 and Ala149 can also inhibit immune response signaling pathways, including induction of interferons [100,101]. On the contrary, deletions in the NS1 gene can attenuate virulence [102]. The NS1 carboxy-terminal PDZ ligand binding motif is also a potential virulence factor. This motif binds cellular PDZ-containing proteins, disrupting a range of cellular signaling pathways [103]. Therefore, monitoring the evolution of the NS1 gene is important for the detection of novel viruses with increased pathogenicity in humans.

H5N1 virus host range

The H5N1 avian influenza virus does not easily infect humans and cannot spread efficiently amongst the human population. Very few suspected cases of human-to-human transmission have been suspected in family clusters of patients, which account for approximately one quarter of total number of cases [40,104]. In most cases, patients probably acquired infection from common-source exposures to poultry or contaminated environment. In some cases, family members in close and unprotected contact with severely ill patient have become ill in the apparent absence of other exposure factors [39, 104,105]. Several studies have demonstrated a lack of human-to-human transmission in high-risk populations such as unprotected health care workers [106,107], therefore it remains unclear whether successful human-to-human transmission has occurred in select cases.

It is noteworthy that more and more cases of avian to mammalian infections with H5N1 virus did naturally occur or were experimentally possible [108,109]. In Indonesia there is a seroprevalence of H5 neutralizing antibodies of up to 20% in the cat population living near poultry markets were H5N1 virus has been circulating [110]. There are also reports in other carnivores like dogs, tigers and leopards, suggesting that H5N1 virus has a potentially broad host range [111,112].

Conclusions

The H5N1 virus which originated in Southern China has spread across 3 continents and is now endemic in many South East Asian countries, with far-reaching effects on public health and local economies. Vietnam and Indonesia have experienced the greatest number of H5N1 outbreaks in both poultry and humans. The Gs/Gd lineage has evolved into multiple novel genotypes through reassortment. Clades are continuously diversifying and requiring further sub-classification. The transduction of H5N1 across South East Asia has occurred through poultry movements and migratory birds. Molecular characterization of H5N1 viruses is an ongoing priority, essential for monitoring antigenic changes, antiviral sensitivity and pathogenicity of novel strains. The pandemic potential of H5N1 virus, 12 years on from its original detection, should not be underestimated.

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References and Notes

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DEUXIEME PARTIE

Sujet de thèse «Etude de la survie du virus H5N1 dans des environnements aquatiques artificiels reproduisant les biotopes naturels du Cambodge, pays d'endémie en zone tropicale»

CHAPITRE 1

Validation d'une technique de concentration, d'identification et de quantification du virus influenza H5N1 dans l'eau

1. Contexte de l'étude

Les canards infectés excrètent de fortes concentrations de virus dans leurs fèces et dans les sécrétions nasales (Webster et al., 1978). La transmission du virus se produit la plupart du temps par contact direct entre oiseaux infectés et essentiellement via les voies respiratoires des hôtes sensibles. Mais le rôle d'une transmission indirecte par l'eau contaminée par les matières fécales a également été confirmé et serait impliqué dans le maintien du VIA chez les canards (Hinshaw et al., 1979; Ito et al., 1995). La contamination des lacs et étangs joue un rôle essentiel comme réservoir de virus de la grippe aviaire A dans l'environnement. L'ingestion ou l'inhalation d'eau contaminée peut être un mode potentiel de contamination humaine. À ce jour, il n'existe que peu de méthodes clairement définies pour extraire et détecter les virus de la grippe dans l'eau. Les concentrations virales sont généralement très faibles dans les eaux de surface ou de consommation, ce qui limite l'efficacité de la détection par PCR. Développer une méthode pour concentrer les virus dans les échantillons est une étape préalable à la détection fiable et efficace de virus présents en faibles concentrations dans l'eau. En se basant sur les principes utilisés pour les entérovirus, la méthode d'adsorption /élution sur des filtres électropositifs semblait bien adaptée pour détecter les virus influenza dans de large volumes d'eau expérimentalement infectés (Roepke et al., 1989) ou d'eau de surface naturellement contaminée (Sivanandan et al., 1991). En alternative, la concentration avec des érythrocytes de poulet (Ito et al., 1995; Khalenkov et al., 2008; Roepke et al., 1989) ou avec du polyéthylène glycol (Markwell and Shortridge, 1982) ont été utilisés sur de petits volumes d'eau du robinet, de lac ou de bassin, parfois en combinaison avec des filtres d'adsorption. Cependant, aucun protocole permettant la concentration,

l'isolement et l'identification de virus de la grippe à partir de larges volumes d'eaux de surface n'a été publié (Roepke et al., 1989).

2. Objectif de l'étude

Le but de cette étude était de développer une méthode permettant la détection de virus de la grippe à partir de gros volumes d'eau de surface, en se basant sur l'adsorption du virus sur des filtres, suivie de leur élution en présence d'une solution de protéines et de leur concentration avec du polyéthylène glycol (PEG). Les objectifs étaient les suivants :

1) Evaluer deux systèmes de filtration: filtration sur laine de verre et cartouche filtrante électropositive NanoCerame.

2) Déterminer l'efficacité de la détection d'un virus représentant de la famille des virus influenza A (souche de référence H1N1 A/PR/8/34).

3) Valider la méthode de concentration pour la détection de souches de virus H5N1dans les eaux provenant de différentes sources artificiellement contaminées.

4) Appliquer la méthode sur des échantillons d'eau de surface naturelle soupçonnés d'être contaminés par le virus H5N1.

3. Résultats de l'étude

Development and Validation of a Concentration Method for the Detection of Influenza A Viruses from Large Volumes of Surface Water
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Development and Validation of a Concentration Method for the Detection of Influenza A Viruses from Large Volumes of Surface Water[▽]

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Contamination of lakes and ponds plays an essential role as a reservoir of avian influenza A virus (AIV) in the environment. A method to concentrate waterborne AIV is a prerequisite for the detection of virus present at low levels in water. The aim of this study was to develop and validate a method for the concentration and detection of infectious AIV from large volumes of surface water samples. Two filtration systems, glass wool and electropositive NanoCeram filter, were studied. The individual effects of filtration-elution and polyethylene glycol (PEG) concentration parameters on the recovery efficiency of the H1N1 strain from 10-liter surface water samples were assessed. An ultimate 1% recovery rate of infectious viruses was achieved with the optimal protocol, corresponding to filtration through glass wool, followed by a viral elution step and then a PEG concentration. This method was validated for the detection of highly pathogenic H5N1 strains from artificially contaminated larger water volumes, from 10 to up to 50 liters, from different sources. The viral recovery efficiencies ranged from 0.01% to 7.89% and from 3.63% to 13.79% with lake water and rainwater, respectively. A theoretical detection threshold of 2.25×10^2 $TCID_{50}$ (50% tissue culture infectious dose) in the filtered volume was obtained for seeded lake waters by M gene reverse transcriptase PCR (RT-PCR). Moreover, the method was used successfully in field studies for the detection of naturally occurring influenza A viruses in lake water in France.

Influenza A viruses belong to the *Orthomyxoviridae* family of negative single-stranded, enveloped RNA viruses. All subtypes of influenza A viruses (H1 to H16 and N1 to N9) have been isolated from wild waterfowls (24, 37). But wild ducks are the main natural reservoir and are generally asymptomatic virus carriers (38). Avian influenza A viruses (AIVs) replicate not only in the respiratory tract but also in the gastrointestinal tract in ducks and are thus shed in high concentrations in the feces (39). Viral transmission occurs most of the time by direct contact between infected birds and essentially the respiratory tract of susceptible hosts. But the role of an indirect waterborne transmission linked to feces-contaminated water has also been confirmed and would be involved in the maintenance of AIVs in ducks (15, 16, 22). AIVs have been isolated from water bodies where waterfowl gather (15, 36, 44) and, moreover, can persist for a long time in water (13, 16). Experimentally, infectious viruses can persist for up to 8 days in bird feces at 22°C (39) and for a few months in cold water (4, 33, 34). All together, these data revealed a mechanism of year-by-year perpetuation of the viruses in the environment where birds breed, especially in cold-climate countries. Contaminated lakes and ponds play essential roles as environmental virus reservoirs. Although most human cases had a history of very close contact with infected poultry, and inhalation of infectious droplets was probably the most common route of infection (3), the oral ingestion or aspiration of contaminated water could be a possible mode of human contamination. To date, there is no clearly defined method to extract and detect influenza viruses in water, although several methods had been employed to determine the viral concentration in water. Based on principles used for enteroviruses, the use of adsorption/elution on electropositive filters was reported and seemed adapted to detect influenza viruses in large volumes of experimentally spiked tap water (27) or naturally contaminated surface water (29). Alternatively, concentration with chicken erythrocytes (16, 18, 27, 29), or with polyethylene glycol (PEG) (22), has been used to concentrate influenza viruses from smaller volumes of tap, lake, or pond water, sometimes in combination with filter adsorption (29). But no recent protocol for the recovery of influenza viruses from surface waters with measured and reported recovery percentages has been published (27).

The objective of the present study was to develop a method for the detection of influenza viruses from large-volume surface water samples, based on the adsorption of the viruses on filters, followed by their elution in the presence of a protein solution and their concentration with polyethylene glycol (PEG). Two filtration systems, glass wool, as used for the detection of enterovirus (2), and NanoCeram electropositive cartridge filter (Argonide), were evaluated. First, a fractional experimental design was conducted to assess individual effects of the filtration system in combination with five filtration-elu-

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tion parameters (filtration flow rate, elution buffer compositions in beef extract and glycine, elution flow rate, and contact time with elution buffer) and two PEG concentration parameters (precipitation and centrifugation times). Recovery efficiencies were determined for a representative virus of the family of influenza A viruses, namely, the H1N1 A/PR/8/34 strain. The concentration method was validated for the detection of highly pathogenic H5N1 strains in artificially contaminated waters from different sources. Moreover, the method was used on natural surface water samples suspected to be contaminated with influenza A viruses in H5N1 outbreak-related places from Cambodia and France.

MATERIALS AND METHODS

Influenza A virus propagation. Influenza A virus subtype H1N1 (A/PR/8/34) was purchased from the American Type Culture Collection (LGC Promochem, Strasbourg, France). Two highly pathogenic avian influenza virus (HPAIV) H5N1 strains, namely, A/HK/156/97 (clade 0) and A/DK/CAM/67F8/2008 (clade 1), were used for experiments performed in France and in Cambodia, respectively. Methods for the propagation of the A/PR/8/34 and A/HK/156/97 influenza viruses on MDCK cells were used in France to prepare inoculums, as previously described (21, 43). In Cambodia, A/DK/CAM/67F8/2008 influenza virus stock was obtained after propagation in specific-pathogen-free (SPF) 9- to 11-day-old embryonated hen eggs, as previously described (12). The viruses were stored at −80°C until further use.

Infectivity assays. Infectivity of influenza H1N1 and H5N1 viruses was determined for experiments conducted in France by using a microtiter endpoint titration, as previously described (21). Infectivity was calculated by the Spearman and Karber method (14) and expressed as the 50% tissue culture infectious dose ($TCID_{50}$) per milliliter, as described in the European standard NF EN 14476 (1). For experiments conducted in Cambodia, an infectivity assay for influenza H5N1 virus was performed on 9- to 11-day-old embryonated chickens eggs, followed by a hemagglutination assay using amnioallantoic fluid as described previously (12, 42). The Reed-Muench method (26) was used to calculate 50% egg infectious dose (EID_{50}). Comparisons were made between the number of EID_{50} and $TCID_{50}$ units for the Cambodian H5N1 virus in order to be able to express results in both units (data not shown).

Water samples and sampling sites. Experiments for development and validation were conducted with water matrix representatives of water bodies where waterfowl gather. A total of 10 to 50 liters of surface waters were collected at approximately 2 m from the waterside. First, to optimize the conditions used with the concentration method, the surface water was sampled from different places in a pond in northern France in autumn. Second, natural water samples were used to validate the method for the detection of experimentally added highly pathogenic H5N1 viruses in water from different sources and to assess the sensitivity of the method. Lake waters were sampled from an ornithological park in northern France and from lakes located in the Kampong Cham and Prey Veng provinces (Cambodia). Rainwater samples were also collected in Cambodia. Finally, natural surface water samples suspected to be contaminated with influenza A viruses from Cambodia and France were used to assess the method for the detection of influenza A viruses from large water volumes. Water samples were previously sampled from the surrounding vicinities of H5N1-infected patients' households in Cambodia in April 2007 (Ponhea Kraek, Kampong Cham province) and December 2008 (Kandal Steung, Kandal province) and stored at −80°C. Water specimens were also collected from Boeung Thom Lake (Kampong Cham) between April and July 2009 and from a wet zone near Prey Trakhob village (Prey Veng) where H5N1 outbreaks occurred in poultry in 2006 (25, 40). Other water samples were collected from three different ponds in France (Dombes region) during the mass migration of birds in autumn 2009, because many birds tested positive for highly pathogenic H5N1 in early 2006.

Filter media and preparation. Glass wool filters were prepared as previously described for the concentration of enteroviruses from water (2, 35). Stainless steel pressure holders (47 mm diameter, 200 ml) were used (Sartorius, France). Fifty grams of oiled sodocalcic glass wool (Saint-Gobain, France) was packed into the holders. NanoCeram electropositive cartridge filters, manufactured by Argonide Corp., Sanford, FL, are ready to use. Pleated filter cartridges are 63 mm wide by 127 mm long. The filter media correspond to multilayer nano alumina fibers, which give them electropositive charges that are dispersed throughout a cellulose and polyester fiber matrix, with a 2 μm average pore size.

Development of the concentration method. The proposed protocol, using a filtration-elution step followed by polyethylene glycol (PEG) concentration, was adapted from existing methods for detection of enteric and influenza A viruses. To obtain an optimal protocol, a fractional experimental design was conducted to evaluate the influence of eight factors on the viral recovery efficiency (Table 1). Four different water samples (A, B, C, and D), corresponding to different sampling dates, were used. Two trials were implemented for each parameter combination. A/PR/8/34 (H1N1) virus stock was added to a final concentration of approximately 1×10^7 $TCID_{50}$ in 10 liters of surface water. After being mixed, a small volume was immediately sampled for the subsequent virus infectivity assay. The seeded water was pumped by a peristaltic pump from a large plastic jerry can through the tested filter at an average flow rate of 10 or 30 liters/h, as previously used (8, 9, 28). Two elution procedures were evaluated for elution of viruses from the glass wool and the NanoCeram cartridge filter with 300 ml and 500 ml of an eluting solution, respectively. The latter consisted of 1.5 or 3% (wt/vol) beef extract (Becton, Dickinson and Company, Le Pont-de-Claix, France) solution (pH 9.5), containing 0 or 0.05 M glycine (Sigma-Aldrich, St. Louis, MO), as previously described (2, 9, 10, 17, 19, 28). Each elution was performed at an average flow rate of 10 or 30 liters/h, as previously used (8, 9, 28). The elution buffer was kept in contact with the filter for 5 or 10 min (corresponding to three successive contact times of 1.5 or 3.5 min with 100 ml elution buffer in the case of the use of the glass wool filter or to a contact time of 5 or 10 min with the entire 500-ml portion of the solution in the case of the use of the NanoCeram filter). The total elution volume was evacuated with air and collected. Filter eluents (corresponding to approximately 400 ml and 500 ml for glass wool and NanoCeram filters, respectively) were neutralized to pH 7 to 7.5 with 1 N HCl. A sample of 5 ml was collected. Viruses present in the filter eluent were concentrated using modifications of the viral concentration method based on PEG precipitation, as previously described (6, 7). A 50% (wt/vol) PEG 6000 (Promega, Madison, WI)-1.5 M NaCl solution was added to obtain a final concentration of 10% (vol/vol), homogenized by shaking, and then incubated at 4°C for 2 h or overnight. The mixture was centrifuged at 10,000 × *g* for 1 or 2 h at 4°C. The supernatant was discarded, and the pellet was resuspended in 3 ml of phosphate-buffered saline (PBS) solution. Each filter eluent and virus concentrate were analyzed separately by plaque assay to determine the infectious virus recovery.

Evaluation of H5N1 virus recovery and detection threshold of the method. The optimal method, corresponding to the factor combination for which the highest level of recovery was previously predicted, was evaluated using two H5N1 strains seeded in natural waters from different sources. H5N1 virus (A/HK/156/97) was seeded into a 10- or 50-liter lake water sample to be tested at high and low final loads of approximately 1×10^7 $TCID_{50}$ and 1×10^3 $TCID_{50}$ per sample, respectively. Three or four trials were conducted for each condition used. The limit of detection of the viral concentration method was assessed. Quantification cycle (Cq) values obtained by reverse transcriptase PCR (RT-PCR) were plotted against the inoculated viral concentration in water. A calibration curve was then built using linear regression. The total number of PCR cycles was 50; it was then checked that a positive result was obtained when a full amplification curve could be observed, which corresponded to detection occurring in less than 42 PCR cycles ($Cq \leq 42$) (data not shown). The detection threshold was thus the viral concentration for which the probability of obtaining a positive result was 0.95. In parallel, eight experiments were performed by seeding 1×10^5 $TCID_{50}$ H5N1 virus A/DK/CAM/67F8/2008 in 10-liter volumes of lake waters sampled in Cambodia. Moreover, to compare recovery values obtained for waters with different physicochemical characteristics, the A/DK/CAM/67F8/2008 virus was seeded into 10 liters of rainwater. Two or three experiments were conducted, with final virus loads ranging from 1×10^3 to 1×10^6 $TCID_{50}$. Virus stocks were used undiluted or diluted in PBS on the day of the experiment and then mixed into the entire volume of water to be filtered. The viral solution was immediately tested with a virus infectivity assay and RT-PCR quantification to determine the seeding viral concentration. A sample of 5 ml was collected just after the elution step to determine the efficiency of virus elution. Elution solutions and PEG concentrates were stored at −80°C until further use. Samples were analyzed by virus titration and by real-time RT-PCR to determine the percentages of virus recovery.

Detection of influenza viruses from environmental samples. Pond and lake waters suspected to be contaminated with AIVs were tested. Ten-liter samples were filtered for validation experiments as described above. Viruses were quantified by RT-PCR.

Viral RNA isolation and quantification. The following protocol was used for the method development, validation of experiments with the A/HK/156/97 H5N1 strain, and detection of virus in environmental samples from France. RNA was extracted from 140 μl of the concentrate using QIAamp viral RNA minikit

TABLE 1. Recovery of infectious H1N1 from large volumes of surface water using different filtration, elution, and PEG concentration conditions

Water sample	Level from factorial design	Parameter used for:								$TCID_{50}$ recovered (%)	
		Filtration		Elution				PEG concn			
				Buffer composition							
		Filter[a]	Flow rate (liters/h)	Beef extract (%)	Glycine (M)	Contact time (min)	Flow rate (liters/h)	Precipitation time (h)	Centrifugation time (h)	Eluate	PEG concentrate
A	1	Glass W	10	1.5	/[b]	10	30	O/N[c]	2	2.3	1.2
	2	NanoC	10	3	0.05	10	30	O/N	1	<0.1	<0.1
	3	NanoC	10	1.5	/	10	10	2	1	<0.1	<0.1
	4	Glass W	10	3	0.05	10	10	2	2	1.0	0.3
	5	NanoC	30	1.5	/	5	30	O/N	1	0.9	<0.1
	6	Glass W	30	3	0.05	5	30	O/N	2	0.6	0.3
	7	Glass W	30	1.5	/	5	10	2	2	1.0	0.7
	8	NanoC	30	3	0.05	5	10	2	1	0.2	<0.1
B	9	Glass W	10	1.5	0.05	5	10	O/N	1	<0.1	0.1
	10	NanoC	10	3	/	5	10	O/N	2	0.8	<0.1
	11	NanoC	10	1.5	0.05	5	30	2	2	<0.1	<0.1
	12	Glass W	10	3	/	5	30	2	1	1.8	<0.1
	13	NanoC	30	1.5	0.05	10	10	O/N	2	0.1	<0.1
	14	Glass W	30	3	/	10	10	O/N	1	5.4	0.5
	15	Glass W	30	1.5	0.05	10	30	2	1	6.4	0.8
	16	NanoC	30	3	/	10	30	2	2	0.1	<0.1
C	1	Glass W	10	1.5	/	10	30	O/N	2	<0.1	<0.1
	2	NanoC	10	3	0.05	10	30	O/N	1	0.1	<0.1
	3	NanoC	10	1.5	/	10	10	2	1	<0.1	<0.1
	4	Glass W	10	3	0.05	10	10	2	2	4.1	1.0
	5	NanoC	30	1.5	/	5	30	O/N	1	0.2	<0.1
	6	Glass W	30	3	0.05	5	30	O/N	2	1.3	0.4
	7	Glass W	30	1.5	/	5	10	2	2	3.9	1.7
	8	NanoC	30	3	0.05	5	10	2	1	<0.1	0.1
D	9	Glass W	10	1.5	0.05	5	10	O/N	1	1.2	<0.1
	10	NanoC	10	3	/	5	10	O/N	2	<0.1	<0.1
	11	NanoC	10	1.5	0.05	5	30	2	2	0.1	<0.1
	12	Glass W	10	3	/	5	30	2	1	2.4	0.4
	13	NanoC	30	1.5	0.05	10	10	O/N	2	<0.1	<0.1
	14	Glass W	30	3	/	10	10	O/N	1	10.5	0.3
	15	Glass W	30	1.5	0.05	10	30	2	1	14.9	3.9
	16	NanoC	30	3	/	10	30	2	2	<0.1	<0.1

[a] Glass W, glass wool filter; NanoC, NanoCeram filter.
[b] /, without glycine.
[c] O/N, overnight.

(Qiagen, Valencia, CA), according to the manufacturer's instructions. The extracted RNA was recovered in 60 μl elution buffer. Quantitative RT-PCRs were performed on a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). Two RT-PCR systems designed for the detection of matrix (M) and hemagglutinin (HA) genes were used separately to detect all subtypes of influenza A viruses and avian influenza viruses H5, respectively. An RT-PCR for each amplification system was performed in a 15-μl reaction mixture, containing 6 mM $MgCl_2$ with 5 μl of extracted RNA, using the Invitrogen SuperScript III Platinum one-step quantitative RT-PCR system and the standard cycling program and TaqMan probes reaction mix protocols recommended by the manufacturer. The sources of the primers and TaqMan probes and their final concentrations used in the present study were as follows: generic M gene (32) and avian H5 gene (30), 500 nM primers and 200 nM probes. A second protocol was used in Cambodia for validation of the experiments with the A/DK/CAM/67F8/2008 H5N1 strain and detection in environmental waters. Viral RNA was extracted from 200 μl of viral concentrate and eluted in 60 μl using the MagNA Pure nucleic acid isolation kit (Roche Diagnostics) on a MagNA Pure Light Cycler instrument (Roche Diagnostics), according to the manufacturer's instructions. One-step RT-PCR using the TaqMan probe was performed on the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) to measure the quantity of the HA gene of the H5N1 avian influenza virus using quantified synthetic RNA. Sequences of the nucleotides and probes used, reaction mixtures using the Quanti-Tect probe RT-PCR kit (Qiagen), and RT-PCR temperature cycling conditions were previously described (41).

The absence of RT-PCR inhibitors was controlled in water samples by addition of an M or H5 gene RNA control (virus-specific internal standard) to each analyzed sample just before RNA amplification.

A standard curve was obtained for each real-time RT-PCR by analyzing 10-fold serial dilutions of viral RNA extracted from the seeding viral concentration for which the infectious titer was determined by infectivity assay. The quantities of viruses in the dilutions were expressed in the $TCID_{50}$ by reference to the logarithmic value of the viral concentration used for viral RNA extraction. The obtained standard curves were used to estimate the quantities of infectious viruses, expressed as equivalent $TCID_{50}$ values, detected in samples. The slope (s) of the standard curve was used to calculate the amplification efficiency (E) of the RT-PCR in conformity with the formula, $E = 10^{(-1/s)} - 1$ (5). Amplification efficiencies from 85% to 115% were considered acceptable.

Virus recovery efficiency. The percent virus recovery was calculated as follows:

$$\text{percent virus recovery} = \frac{(\text{eluent or concentrate})\ \text{titer} \times (\text{eluent or concentrate})\ \text{volume}}{\text{seeded water titer} \times \text{seeded water volume}} \times 100$$

Statistical analysis. Statistical computations and tests were done using S-PLUS statistical software (MathSoft, Seattle, WA). Analysis of variance was performed to evaluate individual effects of the studied factors on virus recovery.

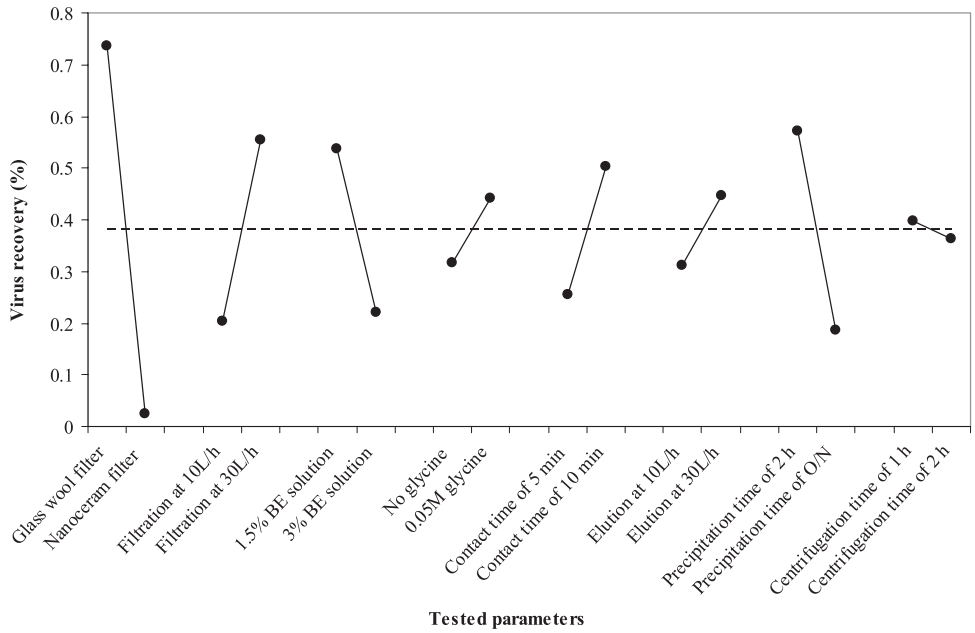

FIG. 1. Individual effects of parameters on infectious H1N1 recovery. The mean recovery by factor level (dot) and global mean recovery (dashed line) are represented. BE, beef extract; O/N, overnight.

Effects were considered significant when P values were <0.05. Linear regression analysis was performed to evaluate the limit of detection.

RESULTS

Optimal concentration method. Recovery rates after adsorption-elution alone and after the subsequent PEG concentration were separately determined for H1N1 virus by titration of the infectious particles on MDCK cells (Table 1). The experimental design enabled the reduction of the experiments to 16 combinations of parameters. Parameter combination 15 (described in Table 1) was identified to be optimal to preserve virus infectivity. Average recoveries of 10.6% and 2.3% of infectious particles were obtained from 10 liters of experimentally contaminated water after elution and after PEG concentration, respectively. The main effects of tested parameters on viral recovery were analyzed and represented in Fig. 1. There was a highly significant difference ($P < 0.05$) in virus recovery by use of glass wool for filtration rather than NanoCeram filters (data not shown). Accordingly, the optimal protocol corresponded to a continuous filtration at 30 liters/h through glass wool, followed by a contact time of 10 min with 300 ml of 1.5% beef extract buffer (pH 9.5) containing 0.05 M glycine and a viral elution step at 30 liters/h, and then a PEG concentration with a precipitation step for 2 h and a centrifugation time of 1 h at 4°C.

Comparison of H5N1 virus recovery from lake water and rainwater and assessment of the sensitivity of the method. The optimal concentration method (described above) was evaluated for H5N1 virus recovery from 10 to 50 liters of seeded lake water and rainwater, and virus concentration efficiencies were compared (Table 2). The amounts of infectious particles added to samples were determined by an infectivity assay. Infectious virus was also recovered from concentrated samples when they were seeded with 1×10^7 $TCID_{50}$ but not when experiments were performed using seeds at 1×10^3 $TCID_{50}$. Consequently, to increase sensitivity for the lower seed levels, virus titers in eluate and concentrate samples were quantified by real-time RT-PCR using a standard curve. For each analyzed sample, comparable Cq values obtained for the RNA control diluted in elution and PEG samples or in water allowed us to check for the absence of RT-PCR inhibitors. In cases of the presence of RT-PCR inhibitors, the results of RT-PCR amplification for 10-fold diluted samples were considered after verification that no inhibition remained after dilution. The overall recovery efficiencies of influenza virus measured in this way ranged from less than 0.01% to 7.89% for 10 to 50 liters of lake waters and from 3.63% to 13.79% for 10 liters of rainwaters. Especially considering the experiments performed with lake waters in France, average recoveries of about 1.9% and 1.0% were obtained by M and H5 gene-specific detection, respectively. Slightly higher values of virus recovery were obtained from 10-liter samples of lake waters than from 50-liter samples. Indeed, no virus was detectable from the 50 liter volume in two out of three trials when 1×10^3 $TCID_{50}$ was seeded and the H5 gene targeted. Moreover, in most experiments in which a low virus concentration was seeded, no virus was recovered in elution samples before the concentration step, whether the H5 or M gene was targeted. The data also suggest that interfering substances in the larger samples affected recovery at the PEG step. In the 10-liter samples for which data are available for both the elution and PEG-concentrated samples, there is a mean 7.7-fold reduction in the percent recovered between the two. For the 50-liter samples, this difference increases to 20.5-fold. Comparatively, an average overall recovery of 0.37% was obtained from 10 liters of lake waters sampled in Cambodia and experimentally seeded with 1×10^5 $TCID_{50}$. Higher values of virus recovery were

TABLE 2. Validation of H5N1 influenza A virus recovery from large volumes of lake water and rainwater

Type of water/country	Seeded virus strain	Date of sampling[e]	Expt no.	Vol of sample (liters)	Gene-specific RT-PCR	$TCID_{50}$ of virus added to sample[c]	$TCID_{50}$ recovered (%)[d]	
							Eluate	PEG concentrate
Lake/France	A/HK/156/97	Oct 2008 (1)	1	10	M	1.71×10^7	34.91	7.89
					H5	6.12×10^6	12.45	3.51
			2	10	M	1.71×10^7	17.60	3.26
					H5	6.12×10^6	6.27	1.21
		Oct 2008 (2)	3	10	M	9.46×10^7	4.37	0.36
					H5	3.38×10^7	4.85	0.22
			4	10	M	9.46×10^7	5.12	1.08
					H5	3.38×10^7	5.27	1.22
		Nov 2008	5	10	M	2.66×10^3	ND[a]	1.88
					H5	1.70×10^3	ND	0.47
			6	10	M	2.66×10^3	ND	1.59
					H5	1.70×10^3	ND	1.36
		Dec 2008	7	10	M	4.94×10^3	ND	2.25
					H5	8.44×10^3	ND	2.59
			8	10	M	4.94×10^3	ND	0.45
					H5	8.44×10^3	ND	ND
		Jan 2009	9	50	M	2.93×10^7	0.20	0.004
					H5	2.52×10^6	ND	0.34
			12	50	M	4.87×10^3	35.52	2.07
					H5	2.21×10^3	ND	0.18
		Feb 2009	10	50	M	5.09×10^6	6.95	0.25
					H5	7.76×10^6	1.80	0.35
			13	50	M	4.30×10^3	34.42	1.90
					H5	2.27×10^3	ND	ND
		March 2009	11	50	M	5.88×10^6	8.61	0.80
					H5	4.70×10^7	0.29	0.02
			14	50	M	3.05×10^3	ND	2.50
					H5	ND[a]	ND	ND
Lake/Cambodia (Kampong Cham)	A/DK/CAM/67F8/2008	April 2009	15	10	H5	1.74×10^5	NT[b]	0.17
		May 2009	16	10	H5	1.74×10^5	NT	0.49
		June 2009	17	10	H5	1.74×10^5	NT	0.09
		July 2009	18	10	H5	1.74×10^5	NT	0.08
Lake/Cambodia (Prey Veng)	A/DK/CAM/67F8/2008	April 2009	19	10	H5	1.74×10^5	NT	1.66
		May 2009	20	10	H5	1.74×10^5	NT	0.04
		June 2009	21	10	H5	1.74×10^5	NT	0.06
		July 2009	22	10	H5	1.74×10^5	NT	ND
Rain/Cambodia	A/DK/CAM/67F8/2008	March 2009	23	10	H5	1.74×10^6	NT	7.57
			24	10	H5	1.74×10^6	NT	8.68
			25	10	H5	1.74×10^5	NT	9.31
			25	10	H5	1.74×10^5	NT	8.79
			26	10	H5	1.74×10^4	NT	3.33
			27	10	H5	1.74×10^4	NT	13.79
			28	10	H5	1.74×10^4	NT	5.98
			29	10	H5	1.74×10^3	NT	ND
			30	10	H5	1.74×10^3	NT	ND
			31	10	H5	1.74×10^3	NT	3.63

[a] ND, not detected.
[b] NT, not tested.
[c] Results obtained by using an infectivity assay.
[d] Results based upon viral quantifications by real-time RT-PCR.
[e] Oct, October; Nov, November; Dec, December; Jan, January; Feb, February.

obtained from rainwaters than from lake waters. The average recovery was about 7.63%.

The detection threshold, corresponding to the amount of infectious particles below which no detection by RT-PCR can be obtained, was evaluated in lake waters by linear regression. Figure 2 shows the M and H5 gene-specific RT-PCR detection results obtained during the experimental scheme to assess the concentration method. A theoretical detection threshold of 2.25×10^2 $TCID_{50}$ in the filtered volume was obtained for the detection of H5N1 virus in lake water by M gene-specific RT-PCR. In addition, a detection threshold of 1.74×10^4 $TCID_{50}$ was obtained by RT-PCR detection of the H5 gene.

Detection of influenza A viruses in environmental waters. No influenza virus subtype H5 was detected in waters collected from places involved in previous outbreaks in Cambodia. However, the concentration method was efficient for the detection

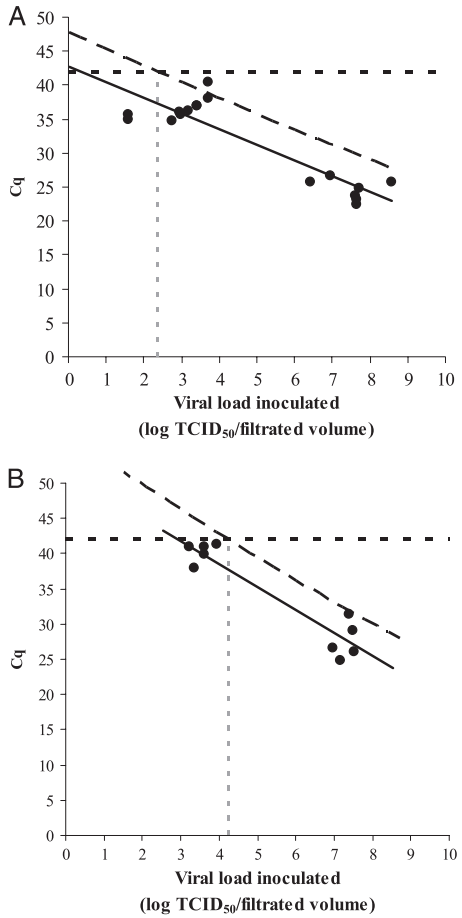

FIG. 2. Assessment of theoretical detection thresholds of the concentration method by M (A) and H5 (B) gene-specific RT-PCR detections from seeded lake water. The linear regression model (solid line) was built with experimental Cq values obtained by RT-PCR and plotted against inoculated viral loads in water (circles). For a given viral load, the Cq value has a 0.95 probability to fall below the diagonal dashed line. A positive detection occurs for Cq values lower than or equal to 42 (horizontal dotted line). The detection threshold, corresponding to the viral load for which a positive detection is obtained with a probability of 0.95, is thus found at the intersection of these two lines.

of influenza A virus in Dombes ponds (France) during fall migration in 2009. AIVs, ranging from 3×10^2 to 9×10^4 $TCID_{50}$ in 10 liters, were detected by M gene RT-PCR in 4 out of 9 water samples, corresponding to samples collected from 1 pond in October and from 3 different ponds in November 2009.

DISCUSSION

During influenza A outbreaks, avian influenza viruses can be isolated from unconcentrated lake water (15, 36, 44). However, since water contamination by AIV possibly occurs at low levels or decreases over time, sensitive methods were needed to detect the presence of these pathogens in natural waters. In contrast to previous studies (16, 18, 22, 27, 29), the present study was conducted using large-volume samples with water matrix representatives of water bodies where waterfowl gather. This type of water sample was used to take into account the impact of water composition (in terms of suspended solids and soluble organic compounds) on virus adsorption to the filter and on flow rate due to filter clogging.

It was important for the concentration method to be appropriate for the detection of infectious viral particles. Therefore, the optimization of the concentration method was performed by evaluating the percent virus recovery from eluent and concentrate samples by using infectious titers obtained by endpoint titration. Moreover, the described procedure was then confirmed as adapted to concentrate infectious H5N1 cultures from large volumes of surface waters. However, the major drawback to the cell culture assay is that it is time-consuming and requires days of incubation, whereas molecular techniques are rapid, highly sensitive, and specific. Therefore, RT-PCR detection methods were preferentially used as the quantification method of determining the equivalent infectious viral load in environmental waters.

The adsorption of viral particles to a membrane is due to electrostatic interactions but depend on both the environmental characteristics and surface properties of the virus (20). Studies showed that charges of influenza viruses above their isoelectric point, which was approximately 5 (11, 23), were negative. Therefore, the natural pH of the treated water, ranging from 7.95 to 8.2 (data not shown), favored virus adsorption on electropositive filters but also persistence of virus in environmental samples (33). When glass wool and NanoCeram filters were compared in this study, glass wool gave significantly higher recoveries of infectious H1N1 virus (Fig. 1; Table 1). Higher recoveries may have been possible with NanoCeram filters if the protocol published after our study was complete had been used (17), but this protocol, which uses two elution steps, has not been tested with influenza. In selection of a two-step concentration procedure to increase sensitivity, it was important to consider the virus concentration efficiency, preservation of infectivity, and compatibility with the filtration and RT-PCR detection methods. Erythrocyte adsorption procedures were often reported as the reference method used for AIVs, when followed by the isolation of influenza viruses in embryonated chicken eggs or tissue culture (16, 27, 29). PCR amplification of the M gene of the influenza virus could be used after this concentration method, but sensitivity was lower than the detection of viral concentrations by embryonated chicken egg isolation (18), probably due to PCR inhibitors produced by red blood cell lysis. In this study, the PEG conditions used were chosen and optimized according to previously described methods for the concentration of infectious enteric viruses from water and vegetables (6, 7). Moreover, the PEG method enabled us to reduce the final volume to 3 ml, which can be assayed by RT-PCR. The results showed the usefulness and concentration efficiency of this method, especially since it was possible to detect virus after PEG concentration, while no virus had been recovered in elution samples from 1×10^3 $TCID_{50}$-seeded waters. However, some weaknesses of the system became evident when virus concentration from larger volumes was attempted or when large amounts of organic matter were present in the water. Humic acid and other organic compounds were also concentrated from water onto filters. These compounds were eluted from the filters along with the virus and formed a precipitate when eluting solution was concentrated by the PEG method. They probably

affected the viral recovery at the PEG step in the larger samples, reducing the recovery percentage in the PEG-concentrated samples and probably interfering with molecular detection of H5 gene when seeding low concentrations and processing 50 liters of lake water. The efficiency of the concentration method was dependent on water characteristics, with more effectiveness for virus concentration using cleaner waters. H5N1 virus recoveries were 5- to 50-fold higher in rainwater than in surface water samples (Table 2). Water characteristics and high levels of soluble organic compounds could significantly affect enteric virus adsorption to electropositive filters (19, 31). Moreover, the method described here is sensitive enough to detect an H5N1 presence in quantities as low as 2.25×10^2 $TCID_{50}$ in 50-liter water volumes by M gene-specific RT-PCR, while erythrocyte methodology enabled the detection of 3.0×10^2 EID_{50} of influenza H1N1 virus in 1 liter of river water (18).

The concentration procedure outlined in this study will facilitate rapid detection of influenza viruses and, moreover, can be used as a quantification method of determining the infectious viral load in environmental waters. Indeed, this system was used successfully in field studies for the detection of naturally occurring influenza A viruses in lake water.

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4. Conclusions de l'étude

Deux systèmes de filtration, laine de verre et filtres électropositifs NanoCeram, ont été étudiés et comparés. Les effets individuels de la filtration, de l'élution et de la concentration par le polyéthylène glycol (PEG) ont été évalués, ainsi que le rendement obtenu sur la souche H1N1 après contamination artificielle d'échantillons de 10 litres d'eau de surface. Lorsque la laine de verre et les filtres NanoCeram ont été comparés dans cette étude, la laine de verre a montré des performances significativement plus élevées. Cette méthode a été ensuite validée pour la détection de souches de virus H5N1 hautement pathogène à partir d'échantillons d'eau artificiellement contaminés sur des volumes importants (10 à 50 litres) et provenant de divers mares et lacs cambodgiens. Un seuil de détection de 2,25 10^2 TCID50 a été obtenue par RT-PCR du gène M. De plus, la méthode a été utilisée avec succès dans les études de terrain pour la détection du virus de la grippe contractée naturellement dans de l'eau d'un lac en France. Au-delà de l'eau, les boues et sols contaminés peuvent jouer un rôle de réservoir pour le VIA. Mais il n'existait aucune méthode spécifique pour détecter du virus H5N1 dans la boue et le sol. L'étude ci-après vise donc à développer et à valider une technique de concentration, d'identification et de quantification du virus influenza H5N1 dans les boues.

CHAPITRE 2

Validation d'une technique de concentration, d'identification et de quantification du virus influenza H5N1 dans la boue

1. Contexte de l'étude

Les virus de la grippe aviaire ont été détectés auparavant dans des réservoirs environnementaux tels que l'eau, les sédiments, les boues, la terre et les fèces (Ito et al., 1995; Lang et al., 2008; Pannwitz et al., 2009; Vong et al., 2008). Les animaux vivant dans des zones où la persistance dans l'environnement est possible sont exposés à des virus de la grippe et à un grand risque d'infection (Breban et al., 2009; Rohani et al., 2009). La plupart des études ont porté sur la caractérisation des virus grippaux isolés à partir des hôtes tels que les oiseaux sauvages et il y a également eu quelques cas d'isolement du virus et la détection de sources abiotiques telles que l'eau et la glace (Stallknecht and Brown, 2009). Plusieurs méthodes ont été employées récemment pour la détection des virus grippaux dans l'eau de surface naturelle (Horm et al., 2011; Khalenkov et al., 2008). Toutefois, la persistance de la grippe aviaire hautement pathogène (IAHP) H5N1 dans le sol ou la boue n'a été que très peu documentée, et des méthodes spécifiques de détection de virus H5N1 dans la boue et des spécimens de sol n'ont pas été décrites. À ce jour, la persistance virale et l'adsorption virale à des sédiments, des boues et des sols ont été principalement étudiées et estimées pour les virus entériques, tels que les entérovirus, le poliovirus et le rotavirus (Goyal and Gerba, 1979; Rao et al., 1986; Sobsey et al., 1980). De nombreuses méthodes ont été décrites dans la littérature et comparées pour la récupération des virus entériques dans les boues (Belguith et al., 2006; Houssin et al., 2007; Hurst et al., 1991; Monpoeho et al., 2001). Pour les virus influenza aviaires, des travaux antérieurs ont montré une détection réussie du virus de la grippe par purification directe de l'acide nucléique à partir d'échantillons de sédiments recueillis dans des lacs en Alaska (Lang et al., 2008). Par ailleurs, l'ARN viral a également été détectés dans des échantillons non concentrés de sol sec et des échantillons de boue (Vong et al., 2008).

Cependant, les échantillons de l'environnement, et en particulier la boue, contiennent des composés organiques et inorganiques (acides humiques, polyphénols et polysaccharides, par exemple), qui sont susceptibles de former des complexes avec les acides nucléiques et ainsi d'inhiber la PCR. Aucune méthode spécifique n'existe pour extraire et détecter les virus de la grippe à partir de l'environnement. Il était donc très important de développer en préalable à toute analyse une méthode simple et fiable qui permette non seulement de détecter de faibles niveaux de virus dans les boues mais aussi d'analyser de larges volumes de boues.

2. Objectifs de l'étude

2.1. Objectifs de la première partie de ce travail

1) Evaluer cinq différents kits commerciaux : QIAamp Viral RNA Mini Kit (Qiagen), PowerSoil™ Total RNA Isolation kit (MOBIO Laboratories), MagNA Pure LC Nucleic Acid IsolationKit (Roche Diagnostics), Trizol et Trizol LS (Life Technologies). Les performances de ces kits pour l'extraction des acides nucléiques du virus H5N1 à partir d'échantillons de boue infectés expérimentalement ont été évaluées de même que le rendement et les seuils de détection.

2) Evaluer une méthode de concentration de virus H5N1 dans les échantillons de boue comprenant une étape d'élution et une étape de concentration avec du polyéthylène glycol (PEG).

2.2. Objectifs de la seconde partie de ce travail

1) Optimiser la méthode de détection du virus de la grippe à partir d'échantillons de boue, adapté à partir des méthodes existantes pour la détection des virus entériques. Analyse comparée de trois solutions d'élution (solution d'extrait de bœuf à 10% (à pH 7), solution d'extrait de bœuf à 3% contenant 0,05 M de glycine (à pH9.5), solution d'extrait de bœuf à 3% contenant 0,1 M borate (à pH 9.5).

2) Evaluer la méthode globale comprenant une étape d'élution et une étape de concentration par PEG.

3) Evaluer et tester la méthode sur des échantillons de boues recueillies lors des épidémies de H5N1 au Cambodge.

3. Résultats de l'étude

3.1 Résultats de la première partie du travail

Direct detection of highly pathogenic avian influenza A/H5N1 virus from mud specimens
Srey Viseth Horm, Nathalie Deboosere, Ramona A. Gutiérrez, Michèle Vialette, Philippe Buchy.
Journal of Virological Methods, 176 (2011) 69– 73.

3.2. Résultats de la deuxième partie du travail

Viral Elution and Concentration Method for Detection of Influenza A Viruses in Mud by real-time RT-PCR
Nathalie Deboosere, Srey Viseth Horm, Alexandre Delobel, Jessica Gachet, Philippe Buchy and Michèle Vialette
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Direct detection of highly pathogenic avian influenza A/H5N1 virus from mud specimens

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A B S T R A C T

Contaminated mud and soil may play roles as reservoirs and sources of transmission for avian influenza A virus. However, the persistence of highly pathogenic avian influenza (HPAI) H5N1 virus in soil or mud has not been well documented, and specific methods of H5N1 virus detection in mud and soil specimens have not been described. The aim of this work was to evaluate the capacities of five different commercial kits and one elution-concentration technique to extract nucleic acids from H5N1 virus and to detect infectious viral particles in experimentally infected mud specimens. The viral RNA detection thresholds for the QIAamp kit, Trizol LS and the MagNA Pure LC kit were 5×10^2 RNA copies per gram of mud. Trizol reagent and the RNA PowerSoil™ kit were unsuccessful in recovering any viral RNA from mud. When the elution-concentration technique was performed prior to nucleic acid extraction, the performance of the MagNA Pure kit increased to a level that allowed the detection of H5N1 nucleic acids in naturally contaminated environmental samples that had previously tested negative after direct extraction using commercial kits. The levels of detection of infectious virus after inoculation into embryonated eggs were higher in concentrates than in eluates.

1. Introduction

Influenza viruses (Family *Orthomyxoviridae*, genus *Influenzavirus A*, species *Influenza A virus*) have been detected previously in environmental reservoirs such as water, sediments, mud, soil, and faeces (Ito et al., 1995; Lang et al., 2008; Pannwitz et al., 2009; Vong et al., 2008). The persistence of viruses in the environment may play a role in virus transmission. Animals living in areas where persistence in environmental reservoirs is possible are exposed to influenza viruses and are therefore at great risk for infection (Breban et al., 2009; Rohani et al., 2009). Most studies have focused on characterising influenza viruses isolated from hosts such as waterfowl; there have also been a few instances of virus isolation and detection from abiotic sources such as water and ice (Stallknecht et al., 2010).

Highly pathogenic avian influenza (HPAI) viruses can cause serious epizootics in domestic birds, but they only occasionally affect wild bird populations. Little is known about the ecology of HPAI H5N1 virus in the natural environment, although it may contribute to virus transmission (World Health Organisation, 2007). Brown et al. (2007) suggested that HPAI viruses could be less adapted than low pathogenic avian influenza (LPAI) viruses to spread by the faecal–oral route in water. However, the persistence of H5N1 virus in soil or mud has not been well documented. During environmental investigations conducted in 3 villages after H5N1 outbreaks in Cambodia, Vong et al. (2008) detected HPAI H5N1 viral RNA in the environment, including in soil and mud specimens. However, specific methods for avian influenza RNA detection, virus concentration and recovery of virus from chemically complex and highly microbiologically contaminated matrices such as mud and soil specimens into culture systems have not been developed and evaluated. In this study, five different commercial extraction kits were assessed for their capacities for recovering H5N1 viral RNA from mud. Additionally, a method of concentrating H5N1 virus from mud specimens, including an elution stage and a polyethylene glycol (PEG) concentration stage, was also evaluated.

2. Materials and methods

2.1. Biosafety statement

All experiments using H5N1 virus were performed within the Biosafety Level 3 (BSL-3) laboratory at the Institut Pasteur in Cambodia (IPC). Experiments involving the use of aquariums containing infected materials were carried out inside isolators within the Animal Biosafety Level 3 (ABSL-3) facility at IPC.

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2.2. Virus

HPAI H5N1 virus A/Cambodia/408008/2005 (GenBank ID: HQ664938–HQ664945) and A/chicken/Cambodia/LC1AL/2007 (GenBank ID: HQ200574–HQ200581) were used to conduct these experiments. Virus stocks were obtained after propagation in Specific Pathogen Free (SPF) 9–11-day-old embryonated hen eggs. The amnio-allantoic fluid (AAF) was harvested 48 h after inoculation and stored at −80 °C until further use.

2.3. Mud and water

Mud and water used for the experiments were collected from a pond in an area of Cambodia where the H5N1 virus has never been detected and where poultry and wild birds do not have access. The absence of virus in the samples was verified by qRT-PCR. For the purpose of this study, two experimental approaches were used. In the first approach, mud was seeded experimentally with H5N1 virus to evaluate the efficiencies of the methods for purifying viral nucleic acids from mud samples. In the second approach, mud was contaminated through immersion into artificially infected water, as described below, to evaluate the efficiency of the elution/concentration method for recovering virus from mud samples.

2.4. Mud contamination in artificial aquatic settings

Artificial aquatic settings were created in aquariums kept inside isolators within an ABSL-3. Two aquariums with capacities of 20 l each were filled with water and mud, allowed to settle for 24 h, and maintained at 22 °C for the duration of the experiments. The water was contaminated on day 0 (D0) with 1×10^7 Egg Infectious Dose 50 (EID_{50}) of HPAI H5N1 virus A/chicken/Cambodia/LC1AL/2007, which corresponds to 3×10^8 RNA copies of the H5 hemagglutinin gene. One gram of mud was collected before virus inoculation (D0) and then daily over 5 days. All specimens collected were stored at −80 °C prior to testing.

2.5. Direct RNA extraction from mud

A total of five commercial kits were compared for their efficiencies for extracting H5N1 viral nucleic acids from mud. Two grams of mud were added to a series of cryotubes, and 10-fold serial dilutions of A/Cambodia/408008/2005 virus (from 10 to 1×10^8 RNA copies) were then added to the tubes.

Method 1 used the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA). Prior to RNA extraction, the mud sample went through a homogenisation step using a MagNA Lyser Instrument (Roche Diagnostics GmbH, Mannheim, Germany) for 2–3 runs of 50 s each at $5000 \times g$. The homogenised mud was then centrifuged. RNA was then extracted from 140 μL of the resulting supernatant according to the manufacturer's instructions.

Method 2 used the RNA PowerSoil™ Total RNA Isolation kit (MOBIO Laboratories, CA, USA). RNA was extracted directly from the 2-g mud sample after a homogenisation step according to the manufacturer's recommendations.

Method 3 used the MagNA Pure LC Nucleic Acid Isolation Kit (Roche Diagnostics). The mud sample was first homogenised using the MagNA Lyser Instrument, and 200 μL of the resulting supernatant was then used for total nucleic acid extraction using the MagNA Pure LC Total Nucleic Acid Isolation Kit with the MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations.

Methods 4 and 5 involved the use of Trizol and Trizol LS (Life Technologies, Gaithersburg, MD), respectively. Viral RNA was extracted directly from two-gram mud samples with 800 μL of Trizol or 600 μL of Trizol LS reagent after a homogenisation step, according to the manufacturer's instructions.

The RNA obtained by each of these 5 extraction methods was eluted in a 50-μL volume of RNase-free water or elution buffer.

2.6. Elution and concentration of virus from mud

An additional method incorporating a virus elution step followed by a concentration step was compared to the direct viral RNA extractions performed with the kits described above. Five-gram aliquots of mud were mixed with a series of 10-fold virus dilutions (10 to 1×10^9 RNA copies) of A/Cambodia/408008/2005 (H5N1). Twenty-five millilitres of 10% beef extract elution buffer (pH = 7) were added to the centrifuge tubes containing mud specimens. The tubes were then placed on a magnetic stirrer and stirred for 30 min at room temperature at a speed sufficient to form a vortex. The tubes were then centrifuged at $10{,}000 \times g$ for 30 min at 4 °C. The supernatants (eluates) were collected, and the solid phase of the mud was discarded. Volumes of 1 or 2 mL of the mud eluates were reserved, and the remaining volumes of mud eluates went through the concentration step. Briefly, a volume of PEG 6000 solution corresponding to 25% of the eluate volume was gently added to each eluate sample and stirred at a very low speed. The mixtures were incubated at 4 °C for 2 h and then centrifuged at $10{,}000 \times g$ for 1 h at 4 °C. The pellets were recovered and dissolved in 1 mL of PBS buffer. The mud eluates and concentrates were either subjected to RNA extraction or inoculated into embryonated hen eggs for infectious virus isolation. RNA extractions were performed with either the QIAamp Viral RNA Mini Kit or the MagNA Pure LC Nucleic Acid Isolation Kit and were then tested by qRT-PCR.

2.7. Environmental samples

Between April 2007 and February 2010, environmental samples were collected during 6 household investigations following reports of human cases of H5N1virus infections or H5N1 virus outbreaks in poultry. A total of 43 mud specimens were obtained from the field, collected in sterile flasks, transported within a few hours to the laboratory at +4 °C and then stored at −80 °C prior to testing.

2.8. Real-time RT-PCR

Viral RNA was detected and quantified using a real-time reverse-transcription polymerase chain reaction (qRT-PCR) targeting the hemagglutinin H5 gene and using synthetic H5 RNA as an internal control and for quantification. The reaction was performed using the primers H5(+)/1544–1563 (5′-CCG CAG TAT TCA GAA GAA GC-3′) and H5(−)/1664–1683 (5′-AGA CCA GCT ACC ATG ATT GC-3′) and the probe H5/1638–1662 (5′-[6-Fam] AGT GCT AGG GAA CTC GCC ACT GTA G [Tamra]-3′). The final reaction mix consisted of 5 μL of RNA with 45 μL of mix containing 5 μL of each primer and probe (3 μM), 1 μL of ROX Reference Dye, 1 μL of the reverse transcriptase enzyme Superscript III/Platinum (Invitrogen), 25 μL of 2× reaction mix, 2.4 μL of 50 mM $MgSO_4$ and 0.6 μL of RNAse-free water. Amplification and sequence detection were then performed in an IQ™5 Real-Time PCR Detection System (BIO-RAD™) thermocycler with a cycling programme of 30 min at 48 °C, 10 min at 95 °C, followed by 3 cycles of 15 s at 95 °C and 1 min at 58 °C, then 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Viral loads were expressed as numbers of copies of RNA per gram of mud. RNA recovery amounts were calculated using the following formula: ([number of RNA copies detected]/[number of RNA copies inoculated] × 100).

Table 1
Recovery amounts (%) of H5N1 viral RNA from experimentally contaminated mud specimens (2 g each) using 5 different commercial nucleic acid extraction methods.

Quantities of viral RNA added to samples	Commercial nucleic acid extraction methods evaluated				
	Qiagen kit	Mobio kit	MagNA Pure LC	Trizol	Trizol LS
10^8 copies	0.03%[a]	0%	0.003%	0%	0.03%
10^7 copies	0.05%	0%	0.01%	0%	0.04%
10^6 copies	0.15%	0%	0%	0%	0.05%
10^5 copies	0.54%	0%	0%	0%	0.2%
10^4 copies	1.81%	0%	0%	0%	3.9%
10^3 copies	19.8%	0%	0%	0%	8%
10^2 copies	0%	0%	0%	0%	0%
10^1 copies	0%	0%	0%	0%	0%

[a] The percentage corresponds to the recovery amount calculated by the following formula: ([number of RNA copies detected]/[number of RNA copies inoculated] × 100).

2.9. Virus isolation in embryonated chicken eggs

All samples that tested positive by qRT-PCR were inoculated into embryonated eggs following standard methods recommended by the World Health Organisation (World Health Organisation, 2002, 2004). SPF 9–11-day-old embryonated chicken eggs were bought from a local supplier. Each specimen was inoculated into 3 eggs. One hundred microlitres were injected into both the amniotic and allantoic cavities. One hundred microlitres of a solution containing streptomycin sulphate, penicillin G, polymyxin B, nystatin, ofloxacin, gentamycin and sulfamethoxazole were also inoculated into the eggs to prevent bacterial and fungal contamination, as described by Khalenkov et al. (2008). The eggs were then incubated for 48 h at 37 °C and chilled overnight at 4 °C. The AAF from each egg was harvested, and standard hemagglutination (HA) tests were performed to confirm the presence of virus. HA tests were performed in 96-well microtitre plates with 0.75% guinea pig red blood cells and serial 2-fold dilutions of AAF. When HA tests were negative, the AAF from each of the three inoculated eggs was pooled and inoculated into a second series of 3 eggs. A maximum of three passages were performed for each sample.

3. Results

3.1. Comparison of the performances of five commercial nucleic acid extraction methods on experimentally infected mud specimens

Both Trizol reagent and the RNA PowerSoil™ Total RNA Isolation kit were unsuccessful in recovering viral RNA from mud, regardless of the quantity of viral RNA mixed with the specimen beforehand (Table 1). These results were verified in a second series of experiments (data not shown). Very low recovery amounts (0.003–0.01%) were obtained using the MagNA Pure LC Nucleic Acid Isolation Kit and only when at least 1×10^7 RNA copies were seeded. However, 1×10^3 to 1×10^8 copies of viral RNA were successfully detected using the QIAamp Viral RNA Mini Kit (recovery amounts ranging from 0.03% to 19.8%) and the Trizol LS method (recovery amounts ranging from 0.03% to 8%). With the best methods, the threshold of avian influenza viral RNA detection was 1×10^3 RNA copies mixed with 2 g of mud specimen.

3.2. RNA detection following the viral elution and concentration method

As described above, two extraction methods were used and compared to purify viral RNA from mud eluates and concentrates. The QIAamp kit did not allow for the detection of viral RNA from mud eluates regardless of the amount of virus inoculated beforehand (Fig. 1). However, 1–2.8% of RNA was recovered using this kit for RNA purification from concentrates from mud experimentally contaminated with 1×10^8, 1×10^7 and 1×10^5 RNA copies (Fig. 1). The RNA detection threshold for the concentrates was 2×10^4 RNA copies/g. With the exception of the recovery amounts obtained for the highest seeded quantity of virus, corresponding to 1×10^9 copies, the results obtained with the MagNA Pure Kit and the QIAamp Kit were comparable for each amount of RNA inoculated to 5 g of mud specimens (Fig. 1). The RNA detection threshold using the MagNA Pure Kit was similar for the eluates and the concentrates (2×10^4 RNA copies/g) (Fig. 1). Overall, the viral loads recovered from eluates and concentrates with the MagNA Pure Kit were not significantly different.

Eluates and concentrates obtained from mud specimens were also tested for the presence of infectious particles. H5N1 virus was successfully isolated from mud eluates when samples were inoculated with a minimum of 1×10^6 copies of viral RNA. A theoretical detection threshold of 2×10^5 RNA copies/g was obtained. This theoretical threshold decreased to 2×10^3 RNA copies per gram when mud was concentrated.

All mud specimens obtained after water contamination in aquariums were also tested by elution and concentration steps followed by extraction using the MagNA Pure LC Nucleic Acid Isolation Kit, as this method appeared to allow RNA purification from eluates and concentrates. Recovery amounts could not be calculated for the samples because the exact amount of virus present in the mud could not be predicted. However, the number of RNA copies detected from eluates and concentrates were compared (Fig. 2). The differences observed between both series of values were not statistically significant (data not shown). The mean numbers of RNA copies detected per gram of mud were 3.3×10^4 in mud eluates versus 1.2×10^4 in concentrates. RNA was detected in eluates and concentrates over the 5 days following water contamination with H5N1 virus. The numbers of RNA copies detected in eluates were 9×10^4 and 3.6×10^3 per gram of mud on days 1 and 5, respectively. On days 3 and 5, 2.3×10^4 and 6.6×10^2 RNA copies per gram

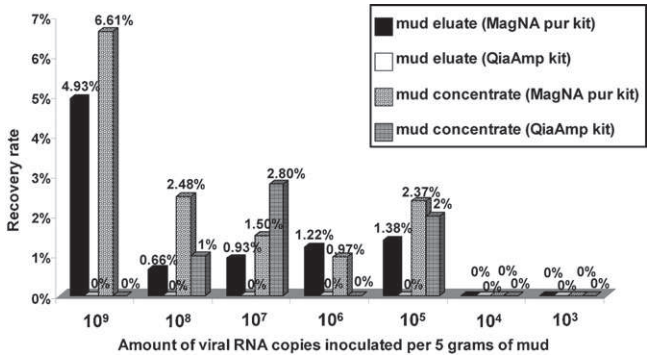

Fig. 1. Recovery rates of H5N1 viral RNA from mud specimens infected experimentally using the elution-concentration method and nucleic acid extraction by MagNa Pure LC Nucleic Acid Isolation Kit and QIAamp Viral RNA Mini Kit.

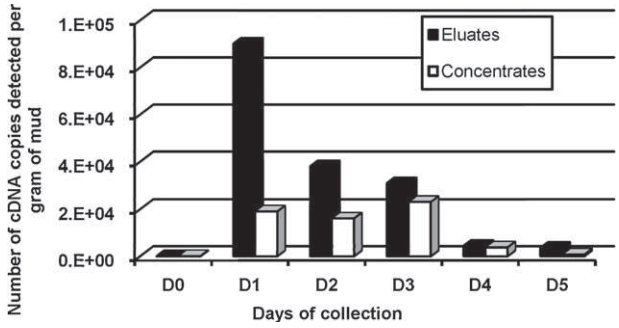

Fig. 2. Detection of H5N1 viral RNA copies in mud experimentally contaminated by contact with seeded water in aquariums.

were detected in concentrates, respectively. However, no infectious particles were recovered from either mud sample eluates or concentrates.

Out of the 43 mud specimens collected from the household investigations performed after H5N1 outbreaks, 7 specimens (16.3%) tested positive by RT-PCR. Direct RNA extraction was performed on 24 samples with the QIAamp kit, and H5N1 viral RNA was detected in two samples (8.3%). Viral loads were 1.1×10^2 and 9×10^2 copies/g. The MagNA Pure LC Nucleic Acid Isolation Kit was used to extract nucleic acids from 19 mud specimens. Only 1 specimen (5.2%) tested positive, corresponding to about 4.5×10^4 copies/g. In addition, 13 samples that were randomly selected from the 36 negative specimens and one sample that had tested positive after extraction with the QIAamp kit were processed using the elution-concentration method as described above and then extracted using the MagNA Pure LC Nucleic Acid Isolation Kit. Using this approach, a total of 5 specimens (35.7%) tested positive in eluates (corresponding to viral loads ranging from 1.1×10^3 to 9×10^4 copies/g). The use of the elution method allowed the detection of viruses in 4 samples that had previously tested negative. A sample that had been quantified as having 9×10^2 copies/g using the QIAamp RNA purification kit also tested positive by eluate using the MagNA Pure Kit (1.1×10^3 copies/g). Only one of the field specimens that tested positive by RT-PCR tested positive after inoculation into eggs.

4. Discussion

The aims of this work were to evaluate the capacities of five different commercial kits and an elution-concentration technique to extract influenza A H5N1 nucleic acids and to recover infectious virus from mud specimens.

Among the five methods tested for direct extraction of H5N1 nucleic acids from mud, the QIAamp Viral RNA Mini Kit and Trizol LS yielded the best recovery amounts. The QIAamp Viral RNA Mini Kit, a general system that uses a silica-gel-based membrane for binding RNA, has been used previously by some authors, including us, to extract avian influenza virus RNA from environmental samples (Dovas et al., 2010; Forster et al., 2008; Vong et al., 2008). However, the recovery efficiency of this method was not evaluated in these studies. Similarly, the performance of Trizol LS, which is based on dissociation of nucleoprotein complexes prior to separation and precipitation of the nucleic acids with chloroform and isopropanol, has never been documented on mud samples, though this technique has been used for influenza RNA extraction from virus propagated in AAF (Chen et al., 2009).

Trizol reagent (non-LS) is recommended for the extraction of RNA from tissues and did not yield detectable amounts of viral RNA from mud, even at high virus concentrations (1×10^8 copies in 2 g of mud). The RNA PowerSoil™ Total RNA Isolation kit, a time-consuming method designed to purify RNA from soil using a phenol/chloroform/isoamyl alcohol solution, was tested repeatedly in this study, but all attempts to extract H5N1 viral RNA from mud failed. According to the information provided by the manufacturer, viral RNA has been isolated successfully from diverse soil types, including compost, manure, estuary sediment and other soils rich in organic content (Mobio, instruction manual). However, these data are not consistent with our experimental results. This difference could be explained by the chemical composition of the mud specimens used in this study, but further investigations will be needed to address this question.

The MagNA Pure LC Nucleic Acid Isolation Kit consists of magnetic glass microsphere particles that bind to nucleic acids and allow the elimination of other elements during washing steps. The nucleic acids are then released in an elution solution. This kit was designed originally for isolation of total viral nucleic acids from mammalian serum, plasma and whole blood. However, it was inefficient at recovering H5N1 RNA from mud specimen supernatants. To the authors' knowledge, attempts using this extraction method on mud or soil have not been reported previously. This magnetic-based system may not be well adapted to bind viral RNA, which, in these experiments, might be trapped in the mud by organic or inorganic particles.

The performance successes of the five methods tested for the direct extraction of H5N1 HPAI viral RNA from mud samples were limited. This could be due to either the small quantities of mud tested (0.5–2 g) or the interferences of some mud components with extraction or amplification steps. Indeed, although the quantity of virus inoculated into the mud increased, the viral load measured did not concomitantly increase. Hypothetically, the same recovery amount should have been observed for each RNA purification method, regardless of the quantity of virus inoculated. The Cambodian sandy soil tested in these experiments contains significant concentrations of PCR inhibitors (in almost 30% of the mud samples tested for PCR inhibitors detection; data not shown), but could also contain various ions and chemicals that interfere with the amplification step in PCR. It is also possible that the mud particles mechanically trap the virus, proteins or nucleic acids and saturate the columns (although the Qiagen kit had better performances than the other methods when used directly on mud) or interact chemically with the reagents used during RNA extraction. Indeed, the performance of the MagNA Pure LC Kit increased significantly after an elution step, which would dilute any PCR inhibitors and hypothetically release some portion of any nucleic acids trapped in the mud (Fig. 1). After the elution and concentration steps, the QIAamp and MagNA Pure Kits showed comparable detection thresholds for the detection of H5N1 viral RNA. However, this might only be the case for the mud and soil used in these experiments, as suggested by partially contradictory results obtained with mud specimens from other origins, which showed much better performances with a magnetic microsphere-based method than with commercial columns (N. Deboosere et al., personal communication). Indeed, the sandy soil found in abundance in the Mekong Basin and in other regions of the world has a very small granulometry, and one could speculate that this soil has a lesser tendency to clog silica-based membranes than soils of different origins and with different compositions. When testing environmental samples, the combination of elution with the MagNA Pure Kit resulted in the detection of viral RNA in 30% of the samples (4/13) that had previously tested negative after direct extraction with the MagNA Pure and QIAamp commercial kits. Of note, 3 out of these 4 samples were collected simultaneously from the same household but in different locations (small pond, large pond, puddle). This represents a significant improvement in the detection of H5N1 environmental contamination. Another important advantage of the elution-concentration technique lies in the fact that it allows the

processing of a large volume of mud (up to 50 g), which should increase the chances of detecting small quantities of virus or RNA. No significant differences were observed when comparing the RNA recovery amounts obtained after elution and after elution concentration. However, the concentration step improved infectious particle detection performance.

Unfortunately, no infectious viral particles could be isolated from either mud eluates or concentrates of mud specimens contaminated by immersion into infected water. This might be explained by factors interfering with virus survival, such as water quality and temperature.

Lang et al. demonstrated that environmental sampling was a valuable technique for assessing the diversity of influenza viruses in specific geographical or environmental locations without the need for more difficult and time-consuming bird sampling and screening of cloacal swabs by real-time PCR or culture. Pannwitz et al. also provided evidence that examination of environmental samples of fresh, wild bird droppings may yield similar avian influenza prevalence ratios compared to pharyngo-cloacal swabs from captured and hunted birds.

The results of this work demonstrate that mud samples tested by a reliable method can be used to efficiently detect HPAI H5N1 virus in natural environments. Although the experimental infections in aquariums were inconclusive, infectious particles were successfully isolated from field ponds. The methods described in this study could therefore contribute to the development of studies aimed at better understanding the persistence of the virus in the environment. The behaviour of the virus outside the host may play a major role in inter-species infections and viral ecology and evolution (Kuiken et al., 2006). Investigations assessing the risks of environmental contamination with HPAI H5N1 virus could help optimise the efforts deployed for disease surveillance and control in countries where H5N1 virus circulates.

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Viral elution and concentration method for detection of influenza A viruses in mud by real-time RT-PCR

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The role of environmental reservoirs in avian influenza virus (AIV) transmission has been investigated during AIV-associated outbreaks. To date, no method has been defined for detection of AIV from mud samples. A procedure using elution and polyethylene glycol (PEG) concentration steps was designed to detect AIV by RT-PCR from 42 g of raw mud, corresponding to 30 g of the solid fraction of mud. RNA was recovered with MagMAX AI/ND Viral RNA Isolation kit (Ambion, Austin, TX). Three elution buffers were studied and viral recoveries higher than 29% were yielded by elution with a 10% beef extract solution (pH 7). The overall method showed that, under some conditions, virus was not detectable in PEG samples, whereas viruses were detected in the elution fractions. PCR curves were improved significantly by running the amplification reaction with a mixture containing a PCR additive for inhibitor removal, such as T4 gene 32 protein (Gp32), although PCR inhibitors from mud were removed partially from PEG samples. A theoretical detection threshold of 5×10^5 RNA copies of H5N1 virus per 30 g of solid mud could be obtained by elution. The overall method has proved successful for detecting H5N1 virus contamination of mud specimens collected during outbreak investigations of avian influenza in Cambodia.

1. Introduction

Influenza viruses are negative single-stranded, enveloped RNA viruses belonging to the family *Orthomyxoviridae*. Wild waterfowls, and particularly ducks, constitute the natural reservoir of all subtypes of avian influenza viruses (AIV) (H1–H16 and N1–N9) (Munster et al., 2007; Webby et al., 2007). In birds, these viruses replicate primarily in the gastro-intestinal tract (Webster et al., 1978). Viruses are shed in large amounts in the faeces and can spread between birds by the faecal–oral route and cause asymptomatic or low pathogenic infection (Webster et al., 1992).

Since 2003, the spread of highly pathogenic avian influenza viruses (HPAIV) H5N1 devastated domestic poultry populations and resulted in the largest and most lethal H5N1 virus outbreak in humans (Neumann et al., 2009; World Health Organisation, 2010). Direct contact between secretions of infected bird and human respiratory mucosa by inhalation of infectious droplets is thought to play a major role in poultry-to-human transmission (Brankston et al., 2007). However, the role of environmental reservoirs in virus transmission, associated mainly with possible faecal contamination of water and also occasionally with mud and sediment samples, has also been investigated during avian influenza-associated outbreaks (Hinshaw et al., 1979; Horm et al., 2011; Lang et al., 2008; Vong et al., 2008; Zhang et al., 2006). The persistence of AIV for quite long periods of time in environmental water has been demonstrated (Halvorson et al., 1983; Ito et al., 1995) and environmental samples would be involved in maintenance of AIV, where waterfowl gather (Hinshaw et al., 1979; Ito et al., 1995; Markwell and Shortridge, 1982). Consequently, several methods have been employed recently for the detection of influenza viruses in natural surface water (Deboosere et al., 2011; Khalenkov et al., 2008). However no method has been defined to extract and detect influenza viruses from environmental complex matrices, such as mud samples.

To date, viral persistence and viral adsorption to sediments, sludge and soil have mainly been studied and estimated for enteric viruses, such as enteroviruses, poliovirus and rotavirus (Goyal and Gerba, 1979; Rao et al., 1986; Sobsey et al., 1980). However, there is no universal method for extracting enteric viruses from sludge (Schwartzbrod, 1991), though a standard method is available in the U.S. (U.S. Environmental Protection Agency, 2003). Numerous methods have been described in the literature and compared for the recovery of enteric virus from sludge (Belguith et al., 2006; Houssin et al., 2007; Hurst et al., 1991; Monpoeho et al., 2001). As viruses are

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adsorbed on sludge particulates (Gerba, 1984), most of these methods are based on elution from the sludge sample by mixing with an alkaline pH solution (9–11.5), followed by a virus concentration step to reduce the volume of eluate before detection.

During the bird migration period of 2005–2006, for the first time, successful detection of influenza viruses by direct nucleic acid (NA) purification was obtained from 2 g sediment samples collected from ponds in Alaska, considered as the intersection of waterfowl migratory routes (Lang et al., 2008). In addition, mud specimens and dry soil swabbing were investigated during and after influenza A virus (H5N1) outbreaks in Cambodia, in 2006 and between 2007 and 2010 respectively, resulting in the detection of viral NA in unconcentrated samples (Horm et al., 2011; Vong et al., 2008). However environmental samples, especially mud, contain organic and inorganic compounds (humic acids, polyphenols and polysaccharides, for example), which are likely to form complexes with nucleic acids and inhibit amplification enzymes. The results of PCR therefore depend on the effectiveness with which the viral extraction technique used removes such compounds. It is important to have a simple and reliable method that can detect low levels of viruses in mud and process mud amounts as large as possible. To date, no procedure has been described in the literature for the elution and the isolation of AIV subtypes from mud.

The objective of the present study was to propose a method for the detection of influenza viruses from mud samples, adapted from existing methods for detection of enteric viruses. It is based on viral elution with a protein solution and concentration with polyethylene glycol (PEG). The method was developed for the detection of a highly pathogenic H5N1 strain (A/HK/156/97) from 42 g of artificially contaminated raw mud, corresponding to 30-g solid fractions of mud. First, three eluents were studied and compared for viral detection from mud. Subsequently, the overall method, including an elution stage and a PEG-concentration stage, was evaluated. The AIV were quantified by an influenza matrix gene-specific RT-PCR. Then, the method was tested on mud specimens collected during H5N1 outbreak investigations in Cambodia.

2. Materials and methods

2.1. Virus strain

HPAIV A/Hong Kong/156/97 (H5N1) (GenBank ID: AJ289874) was used to conduct these experiments (Claas et al., 1998; Subbarao et al., 1998). This virus was first isolated from a patient living in Hong Kong in 1997 and derived by adaptation of egg-passage to MDCK continuous cell line (Madin Darby Canine Kidney cell). The strain was kindly provided by Dr. Jean-Claude Manuguerra (Institut Pasteur-Paris, France). Virus stocks were obtained after propagation on MDCK, as previously described (Lénès et al., 2010).

2.2. Mud samples

To optimize the conditions of the elution and concentration method, mud samples were obtained from an ornithological park from Northern France, to be representatives of places where waterfowl gather. Samples were collected at approximately 2 m from waterside at different places in the pond in February and March 2009. Previous experiments were conducted to determine total solid fraction of mud samples. Briefly, a raw mud portion was placed in a centrifuge bottle and weighted. The mud sample was centrifuged at 2500 × *g* for 10 min at 4 °C and supernatant was discarded. Total solids were determined by weighing. The raw mud weight was calculated to obtain 30 g of total solids.

In addition, in response to the notification of human H5N1 virus infections in Cambodia between April 2007 and February 2010, mud specimens were collected in the environment of the index cases' households and in the surrounding vicinity when poultry mortality was reported. 14 samples of 5-*g* each were randomly selected for the purpose of this study to assess the method.

2.3. Evaluation of elution buffers for H5N1 virus recovery from mud

The experimental design for influenza virus elution from solids was based on the standard method for the recovery of enteric viruses from sludge described in the USEPA 625/R-92/013 (U.S. Environmental Protection Agency, 2003). Experiments were conducted to compare viral recoveries by elution from artificially contaminated mud, using three eluting solutions that had previously been described for detection of enteric viruses (Albert and Schwartzbrod, 1991; Houssin et al., 2007; U.S. Environmental Protection Agency, 2003). Eluting solution 1 corresponded to 10% (w/v) beef extract (Becton, Dickinson and Company, Le Pont-de-Claix, France) solution (pH 7); solution 2 corresponded to 3% (w/v) beef extract solution (pH 9.5), containing 0.05 M glycine (Sigma–Aldrich, St. Louis, MO, USA); and solution 3 corresponded to 3% (w/v) beef extract solution (pH 9.5), containing 0.1 M borate (Sigma–Aldrich). Three trials were implemented for each elution condition tested. Regarding artificial contamination of samples, although $AlCl_3$ is used for enhancing virus adsorption in the USEPA method (U.S. Environmental Protection Agency, 2003), it was not used in this study because more than 99.6% adsorption of H5N1 was achieved with simple stirring (data not shown). Therefore virus H5N1 stock containing approximately 6×10^8 RNA copies (corresponding to approximately 2×10^6 $TCID_{50}$) was added to a 42 g raw mud. The viral stock was also sampled for RT-PCR quantitation and virus infectivity assay. Then, viruses were adsorbed to mud by stirring with a magnetic stirrer for 30 min. The mud sample was centrifuged at 2500 × *g* for 10 min at 4 °C. Supernatant was cleared and a small volume was immediately quantified by RT-PCR to confirm that no or less than 0.4% viral RNA could be detected in supernatant in each experiment (data not shown). The pellet, corresponding to approximately 30 g of mud solid fraction, was dispersed in 250 ml of each eluent. The suspension was stirred with a magnetic stirrer for 30 min at room temperature, at a speed sufficient to develop a vortex. Then, the mixture of the pellet and the eluent was centrifuged at 10,000 × *g* for 1 h at 4 °C and its supernatant was collected. The mud eluent (corresponding to approximately 250 ml) was neutralized with 1 N HCl solution, resulting in a final elution solution with a pH ranging from 7 to 7.5. Viruses recovered in elution solutions were quantified by RT-PCR.

Percentage of virus recovery was calculated as following:

$$\text{Percent virus recovery} = \frac{\text{number of RNA copies detected}}{\text{number of RNA copies inoculated}} \times 100$$

2.4. Detection threshold of the elution and concentration method

The detection threshold of the complete method for the detection of influenza viruses from mud samples was evaluated. Virus stocks were diluted in appropriate volumes of Dulbecco's Phosphate Buffered Saline (DPBS) to reach virus loads ranging from approximately 6×10^5 to 6×10^8 RNA copies (corresponding to approximately 2×10^3 to 2×10^6 $TCID_{50}$) per 42-g raw mud. (6×10^5 RNA copies/30 g of solid mud corresponded to the lowest quantities of viral RNA theoretically detectable in 250-ml elution fractions, due to the viral RNA isolation conditions and the detection threshold of the used RT-PCR method). The viral stock dilutions were also tested by RT-PCR quantitation and by infectious virus titration. Viruses were eluted from mud samples as described above. Supernatants were removed after the viral adsorption and

elution steps, respectively, and sampled for RT-PCR quantitation. Then, viruses present in the elution solution were concentrated using the viral concentration method based on PEG precipitation, as described previously (Deboosere et al., 2011). A 50% (wt/vol) PEG 6000 (Promega, Madison, WI)/1.5 M NaCl solution (1:4) was added before homogenization by magnetic stirring, then incubated at 4 °C for 2 h. The mixture was centrifuged at 10,000 × *g* for 1 h at 4 °C. The supernatant was discarded and the pellet was dissolved in 10 ml of DPBS. Viral RNAs present in elution solutions and PEG-concentrates were quantified by RT-PCR. Three trials were implemented for each viral load tested.

The limit of detection of the viral elution and concentration method was assessed. Quantitation cycles (C_q) obtained by RT-PCR were plotted against inoculated viral concentration in mud. A calibration curve was then built using linear regression. The total number of PCR cycles was 50; it was then checked that a positive result was obtained when a full amplification curve could be observed, which corresponded to detection occurring in less than 42 PCR cycles ($C_q \leq 42$) (data not shown). The detection threshold was the viral concentration for which the probability of obtaining a positive result was 0.95.

2.5. Viral RNA isolation and real-time RT-PCR

For the optimization of the method, RNA was extracted from 300 μl using MagMAX™ AI/ND Viral RNA Isolation kit (Applied Biosystems/Ambion, Austin, TX), according to the manufacturer's instructions. The extracted RNA was recovered in 50 μl elution buffer. The MagMAX Kit uses microspherical paramagnetic beads to bind nucleic acid and is designed for purification of avian influenza and Newcastle disease viral RNA from pharynx/tracheal and cloacal swab samples. The use of QIAamp Viral RNA Mini Kit® (Qiagen, Valencia, CA, USA) has been also evaluated to purify viral RNAs: RNA recovery percentages achieved with the MagMAX Kit were significantly more reproducible and higher than these obtained with QIAamp Kit (data not shown).

A real-time reverse-transcription polymerase chain reaction (qRT-PCR) targeting matrix M gene was used for the detection and quantitation of viral RNA from all subtypes of influenza A. The reaction was performed using the primers M(+)/25 (5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′) and M(−)/124 (5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′) and the probe M/64 (5′-[6-Fam] TCA GGC CCC CTC AAA GCC GA [Tamra]-3′) (Spackman et al., 2002). The final reaction mixture contained 5 μl of extracted RNA with 15 μl of mix using Superscript III Platinium One-Step Quantitative RT-PCR system (Invitrogen) and 1 μl of each primer (500 nM), 0.16 μl of probe (200 nM), 0.8 μl of the reverse transcriptase enzyme Superscript III RT/Platinum Taq mix (Invitrogen), 10 μl of 2× reaction mix, 1.2 μl of 50 mM $MgSO_4$, 0.2 μl of non-acetylated Bovine Serum Albumine (BSA) (Invitrogen) and 0.64 μl of RNAse-free water. Amplification and sequence detection were then performed in a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) thermocycler using a cycling programme of 15 min at 50 °C, 2 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 30 s at 60 °C. For PCR inhibitors removal, 2 μg T4 gene 32 protein (Gp32) (Roche Diagnostics) were added to the mix reaction, before RNA amplification (Kreader, 1996; Monpoeho et al., 2000).

For the detection of AIV in environmental mud collected in Cambodia, viral RNA was extracted using the MagNA Pure LC Nucleic Acid Isolation Kit (Roche Diagnostics) and then detected using a qRT-PCR targeting the hemagglutinin H5 gene as previously described (Horm et al., 2011). Amplification and sequence detection were performed in an IQ™5 Real-Time PCR Detection System (BIO-RAD™). Sequences of nucleotides and probes used, reaction mixtures and RT-PCR temperature-cycling conditions were chosen as recommended by the World Health Organisation (World Health Organisation, 2007).

Synthetic RNAs as virus-specific internal controls were used for quantitation and for control of the absence of RT-PCR inhibitors in mud samples. Viral loads were expressed as numbers of RNA copies. The slopes (s) of the standard curves were used to calculate the amplification efficiency (E) of the RT-PCR in conformity with $E = 10^{-1/s} - 1$ (Bustin et al., 2009). Amplification efficiencies from 85% to 115% were considered as acceptable.

Table 1
H5N1 recovery from mud samples using different elution buffers.

Elution buffer	RNA copies of virus added to sample	RNA copies recovered in eluate (%)
10% Beef extract (pH 7.0)	6×10^8	39.6
	6×10^8	39.7
	8×10^8	29.0
3% Beef extract 0.1 M borate (pH 9.5)	6×10^8	9.1
	6×10^8	3.3
	8×10^8	7.5
3% Beef extract 0.05 M glycine (pH 9.5)	6×10^8	9.6
	6×10^8	5.7
	8×10^8	6.7

2.6. Infectivity assays and virus isolation

Infectivity of influenza H5N1 virus was determined by using a microtiter endpoint titration, as previously described (Lénès et al., 2010). Endpoints were reported as 100% monolayer destruction. Infectivity was calculated by the Spearman and Kärber method (Hamilton and Thurston, 1977) and expressed as 50% Tissue-Culture Infectious Dose ($TCID_{50}$/ml), as described in the European standard NF EN 14476 (AFNOR, 2005).

All environmental samples that tested positive by qRT-PCR were inoculated into embryonated eggs for virus isolation, following standard methods recommended by the World Health Organisation (World Health Organisation, 2002, 2004) and as described previously (Horm et al., 2011).

3. Results

3.1. Comparison of three different elution buffers

Three elution buffers, corresponding to 10% beef extract buffer (pH 7.0), 3% beef extract/0.05 M glycine solution (pH 9.5) and 3% beef extract/0.1 M borate solution (pH 9.5), were evaluated (Table 1). Average recoveries of 36.1%, 6.6% and 7.3% viral RNA from mud specimens were obtained by elution with the three previous elution buffers, respectively. Accordingly, the optimal elution buffer, namely 10% beef extract buffer (pH 7.0), was used for concentration and detection of influenza A viruses in subsequent experiments.

3.2. Viral RNA recoveries using the elution and concentration method

The elution step, using a 10% beef extract solution, and the polyethylene glycol (PEG) concentration were evaluated for H5N1 virus recovery from 30-g mud solids contaminated experimentally with different virus loads (Table 2). No virus was detectable in concentrated samples from mud in two trials out of three when 5×10^5 RNA copies were used to contaminate mud, whereas viruses were detected in the elution buffer. Moreover, concentrated samples needed to be 10-fold diluted before RT-PCR to dilute PCR inhibitors and enable virus detection. Protein-based additives, such as BSA

Table 2
H5N1 recovery from mud samples.

RNA copies of virus added to sample	RNA copies recovered (%)	
	Eluate	PEG concentrate
8×10^8	29.0	1.7
	39.6	33.0
6×10^8	39.7	9.8
	54.0	3.5
8×10^6	31.9	39.8
	36.6	22.5
	43.9	<5.2
6×10^5	45.8	<5.2
	66.3	10.4

and Gp32, have been also used and compared to eliminate PCR inhibition (data not shown). The use of Gp32 improved PCR curves and increased fluorescence intensity (Fig. 1), but inhibition controls still showed the presence of RT-PCR inhibitors in 10-fold diluted PEG-sample extracts. Therefore, the detection threshold, corresponding to the amount of viral RNA below which no detection by RT-PCR can be obtained, was evaluated in eluted mud samples by linear regression. Fig. 2 shows the M gene-specific RT-PCR detection results obtained during the experimental scheme to assess the elution method. A theoretical detection threshold of 5×10^5 RNA copies of H5N1 virus per 30 g of solid mud was obtained in elution fraction. The elution method is thus sensitive enough to detect viral presence at concentrations as low as 1.6×10^4 RNA copies/g in 30-g mud solid amounts using M gene specific-RT-PCR.

Eluates and concentrates obtained from mud specimens were also tested for the presence of infectious particles. This method was unsuccessful in obtaining H5N1 virus titration from these samples, which showed cytotoxicity phenomena on MDCK cells.

3.3. Detection of influenza A viruses in environmental samples

Mud samples, collected during outbreak investigations in Cambodia and possibly contaminated by AIV, were tested using adapted volumes of 10% beef extract elution and PEG solutions. Out of 14 mud samples, 5 specimens were tested positive in eluates. The use of the elution method allowed the detection of viruses in 4 samples that had previously tested negative by using commercial nucleic acid extraction methods for direct RNA extraction from mud samples (Horm et al., 2011). Only one of the field specimens that tested positive by RT-PCR was able to grow in eggs after inoculation with the eluate.

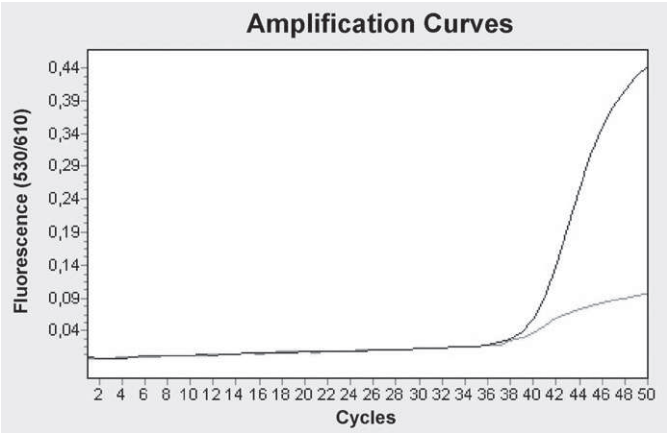

Fig. 1. Comparison of intensity and amplification quality in a mud eluate. This extract, containing 3×10^1 viral RNA copies in 5 µl, was tested by RT-PCR in the presence (in black) and absence (in gray) of Gp32.

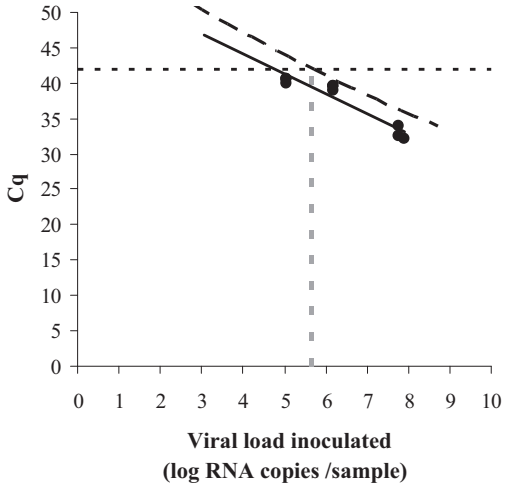

Fig. 2. Assessment of the theoretical detection threshold of the elution method by M gene-specific RT-PCR detections from virus seeded mud. Linear regression model (continuous line) was built with experimental C_q obtained by RT-PCR plotted against inoculated viral load in mud (circles). For a given viral load, the C_q value has a 0.95 probability to fall below the dashed line. A positive detection occurs for C_q values lower than or equal to 42 (horizontal dotted line). The detection threshold, corresponding to the viral load for which a positive detection is obtained with probability 0.95, is thus found at the intersection of these two lines.

4. Discussion

The objective of this study was to develop an efficient method for the detection of influenza A viruses from mud. A variety of protocols have been described regarding elution and concentration method suitable for detecting enteric viruses in sewage sludge (Belguith et al., 2006; Houssin et al., 2007; Hurst et al., 1991; Monpoeho et al., 2001). Most of them are based on an extraction step which enables to break the link between viruses and sludge particles (Schwartzbrod, 2000). Enteric viruses have a natural affinity to adhere to organic material (Hurst et al., 1978). The adsorption of viral particles to a surface or to mud particles is mainly due to electrostatic interactions between the viral capsid and the surface itself. However, the phenomena of virus adhesion and thus their elution depend both on environmental characteristics and surface properties of the virus (Langlet et al., 2008). Charges of viruses are negative above their isoelectric point (pI) and positive below. The pI value for influenza viruses, corresponding to the pH value at which the virus presents no charge, ranges from 4.0 to 7.0 (Michen and Graulet, 2010). The composition of the eluent solution, generally alkaline pH solutions, enriched with protein and minerals, is very important. High pH solutions appear to create strong electrostatic repulsive force between negatively charged soil and virus particles, causing virus desorption (Rao et al., 1986). Moreover, these solutions change environmental conditions by presence of proteins which compete with viruses for adsorption on the binding sites. In this study, the natural pH of the studied mud, ranging from 7.7 to 8.0 (data not shown), favoured virus adsorption on mud, but also probably persistence of viruses in environmental samples. Moreover, the pH of the mud/eluent mixture must be higher than the isoelectric point of viruses to release them into the liquid phase. Therefore, the use of buffer with the highest proteins content, i.e. 10% beef extract (pH 7), significantly increased the recovery percentage of virus H5N1 (Table 1). In addition, the ratio of the volumes of sludge and eluent, ranging from 1/10 to 1, and homogenization methodology, such as magnetic stirring, mechanical agitation or

sonication, seemed to have an effect on the elution effectiveness of enteric viruses (Schwartzbrod, 2000). Time contacts between eluent solution and mud ranged from 1 to 60 min. In this study, the ratio mud (total solid weight)/eluent of approximately 1/10 (w/v) and homogenization by magnetic stirring for 30 min were chosen. A second-step concentration procedure was selected to increase sensitivity and it was important to consider the virus concentration efficiency and compatibility with RT-PCR detection methods. The PEG protocol was described for the concentration of influenza viruses from water (Deboosere et al., 2011), for which it was found to be an adapted concentration step to reduce the final volume, which can be assayed by RT-PCR. Unfortunately, this concentration step was not always efficient to recover viruses in this study. Indeed, no viral RNA could be detected (in two trials out of three) from mud contaminated with approximately 6×10^5 RNA copies, while viruses were recovered in elution samples. This could be due to the presence of PCR inhibitors in mud specimens. When large amounts of organic matter were present in the mud, humic acid and other organic compounds were eluted from the mud along with the virus and formed a precipitate when the eluting solution was concentrated by PEG. They probably interfered with molecular detection of M gene and inhibited detection when the virus was present at the lowest concentration. Some actions can alleviate or overcome PCR inhibition, some of which have already been mentioned with respect to specific inhibitors. Protein-based additives, such as BSA and Gp32, can be added to a PCR, either to act as a substrate for protease activity or to bind inhibitors. Humic acid inhibitors or extracts from faeces and marine water could be accommodated in the PCR, when BSA (400 ng/μl) or Gp32 (150 ng/μl) were included in the reaction (Kreader, 1996). A balance was achieved in both reducing inhibition and enhancing the PCR by use of these additives. In this study, the use of Gp32 (100 ng/μl in mix reaction) improved PCR curves and increased fluorescence intensity (Fig. 1), as previously described (Monpoeho et al., 2000).

Additional experiments using this elution–concentration method were performed in Cambodia to detect avian influenza viral RNA from artificially contaminated muds (Horm et al., 2011). The RNA detection threshold (2×10^4 RNA copies/g) was confirmed and was similar for both the eluates and also the concentrates, that was in disagreement with the present study, where detection in the PEG samples was rarely successful. This may be possible due to either the small quantities of mud tested by Horm et al. (0.5–2 g), or the physico-chemical characteristics of mud collected from Cambodia, or the used molecular detection tools targeting H5 gene (instead of M gene).

In this study, the use of a magnetic microsphere-based method to extract viral RNA from mud significantly increased the nucleic acid detection present in eluates and precipitates. Results showed better performances with the MagMax Kit than with the QIAamp Kit using silica-based columns, which may possibly be clogged by mud particles. The microspherical paramagnetic beads used in the MagMax™ AI/ND Viral RNA Isolation kit can be fully dispersed in solution, allowing nucleic acid binding and probably removal of some inhibiting particles. Indeed, another magnetic microsphere-based kit, namely MagNA Pure LC Nucleic Acid Isolation Kit (Roche), could be successfully used in combination with the elution–concentration method to detect H5N1 virus contamination of mud specimens from Cambodia.

The elution procedure, involving a 10% beef extract solution at pH 7, was recommended for the recovery of enteric viruses by the USEPA (U.S. Environmental Protection Agency, 2003), and has also proven to be adapted, quite rapid, sensitive and reproducible for the detection of influenza viruses. However further investigations could be carried out, for example to optimize beef extract concentration over a broader range. The method outlined in this study, either with or without the PEG-concentration step, could be used to analyze large mud amounts for the detection of influenza viruses in environmental samples. Thus the elution–concentration method could be used in association with another comparable nucleic acid extraction kit and with other molecular detection tools to detect successfully H5N1 virus RNA in environmental mud specimens. Moreover, the use of elution resulted in the detection of viral RNA in 4 samples that had previously tested negative after direct extraction with different commercial kits (Horm et al., 2011). However, these results should be confirmed by the analysis of a larger number of samples, since only 13 were tested.

Face to the lack of scientific knowledge on many aspects of the ecology and environmental properties of HPAIVs, in particular H5N1, this method could be used to understand the year-by-year perpetuation of the virus and its survival in the environment.

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4. Conclusions de l'étude

Parmi les cinq méthodes testées pour l'extraction directe de l'acide nucléique viral à partir de la boue, le QIAamp Viral RNA Mini Kit et le Trizol LS ont donné les meilleurs résultats. Lorsque la technique d'élution/concentration a été appliquée préalablement à l'extraction des acides nucléiques, la performance de kit MagNA pure LC a augmenté à un niveau qui a permis la détection de l'acide nucléique du virus H5N1 dans des échantillons de boue contaminés naturellement et qui avaient été préalablement testés négatifs après l'extraction directe à l'aide de kits commerciaux. Les niveaux de détection de virus infectieux après inoculation dans des œufs embryonnés ont été plus élevés dans les concentrés que dans les éluats de boue.

Parmi les 3 solutions d'élution testées, un taux rendement d'environ 30% a été obtenu lors de l'utilisation d'une solution d'extrait de boeuf à 10% (pH 7). Dans certaines conditions, aucun virus n'a pu être détectée dans des concentrés de boue (concentration par le PEG), tandis que les virus ont été détectés dans les fractions d'élution. Un seuil de détection théorique de 5×10^5 copies d'ARN du virus H5N1 par 30 g de boue solide a pu être défini.

Globalement, la méthode d'élution/concentration a fait ses preuves dans la détection de la contamination du virus H5N1 dans des prélèvements boues recueillis au cours des enquêtes épidémiologiques au Cambodge.

Notre étude montre que pour mieux détecter le virus H5N1 dans la boue, l'élution (avec la solution d'extrait de bœuf 10% (pH 7) puis la concentration (avec du PEG) doivent être réalisées préalablement à l'extraction de l'acide nucléique (soit avec le kit MagNA pure LC, soit avec le kit Qiagen) et à la qRT-PCR.

Les méthodes validées ci-dessus (Chapitres 1 et 2) ont été utilisées dans les analyses des prélèvements d'eau, de boue de l'environnement naturel dans les investigations épidémiologiques des foyers d'épidémie du H5N1 au Cambodge (Chapitre 3) et dans des expériences réalisées dans des biotopes artificiels dans le cadre de notre étude (Chapitre 4).

CHAPITRE 3

Survie du virus influenza H5N1 dans l'environnement

1. Contexte de l'étude

Il a été précédemment démontré que le virus H5N1 pouvait persister dans l'eau (Domanska-Blicharz et al., 2010; Stallknecht and Brown, 2009) et que l'ARN viral pouvait être détecté dans des échantillons environnementaux (Iglesias et al., 2010; Lebarbenchon et al., 2010; Van Kerkhove et al., 2011), y compris dans les alentours des foyers H5N1 au Cambodge (Vong et al., 2008). Dans ce pays, les cas humains d'infection par le virus H5N1 on principalement fait suite à un contact direct avec des volailles infectées (Buchy et al., 2007), bien que études séro-épidémiologiques aient également identifié la baignade dans les mares comme un autre facteur de risque de contamination humaine (Cavailler et al., 2010; Vong et al., 2009). Ceci est cohérent avec les données rapportées par les pays voisins, qui suggèrent également que l'exposition au virus H5N1dans des environnements contaminés (eau souillée, installations d'abattage des volailles, engrais à base d'excréments, litières), sans contact direct avec des volailles infectées est associée à un risque élevé d'infection humaine (de Jong et al., 2005; Food and Agriculture Organization; Kandun et al., 2010; Kandun et al., 2006; WHO, 2007). Le rôle exact de l'environnement dans la transmission du virus H5N1 reste cependant mal compris. Peu d'auteurs ont décrit la survie du virus H5N1 dans l'eau, le sol ou les surfaces en conditions contrôlées au laboratoire, avec des températures allant généralement de 0 à 25 °C. Mais il existe encore moins de données relatives à la persistance du virus dans les milieux naturels où des épizooties se produisent régulièrement, et entre autres dans les pays tropicaux où les températures moyennes peuvent atteindre plus de 35 °C à l'ombre.

2. Objectifs de l'étude

Le but est d'étudier diverses sources environnementales entourant les zones d'épizooties comme réservoirs potentiels de virus H5N1. Etudier s'il y a contamination par le virus H5N1

des divers prélèvements environnementaux (eau, boue, sol, poussière, plantes et animaux aquatique,..) collecté dans ces zones.

3. Résultats de l'étude

Environment: a potential source of animal and human infection with influenza A (H5N1) virus.
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Original Article

Environment: a potential source of animal and human infection with influenza A (H5N1) virus

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Background Very little is known regarding the persistence of highly pathogenic avian influenza H5N1 viruses in natural settings during outbreaks in tropical countries, although environmental factors may well play a role in the persistence and in the transmission of H5N1 virus.

Objective To investigate various environmental compartments surrounding outbreak areas as potential sources for H5N1 virus transmission.

Methods Environmental specimens were collected following outbreaks of avian influenza in Cambodia between April 2007 and February 2010. The methods used to concentrate H5N1 virus from water samples were based either on agglutination of the virus with chicken red blood cells or on adsorption on glass wool, followed by an elution-concentration step. An elution-concentration method was used for mud specimens. All samples that tested positive by real-time RT-PCRs (qRT-PCRs) targeting the HA5, M and NA1 genes were inoculated into embryonated hen eggs for virus isolation.

Results Of a total of 246 samples, 46 (19%) tested positive for H5N1 by qRT-PCRs. Viral RNA was frequently detected in dust, mud and soil samples from the farms' environment (respectively, 46%, 31% and 15%). Samples collected from ponds gave a lower proportion of positive samples (6%) as compared to those collected from the farms (24%). In only one sample, infectious virus particles were successfully isolated.

Conclusion During H5N1 virus outbreaks, numerous environmental samples surrounding outbreak areas are contaminated by the virus and may act as potential sources for human and/or animal contamination.

Keywords Cambodia, environment, H5N1 virus, influenza, outbreaks, transmission risk.

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Introduction

Poultry infected with avian influenza viruses (AIV) usually shed large numbers of viral particles in their faeces, saliva and nasal discharge,[1,2] which can lead to the contamination of environmental components such as water, pond sediment, mud and soil, as shown in various experimental[3–5] and field studies.[6–8] Previous studies focusing on live bird markets also showed that several AIV subtypes could be isolated from environmental swabs collected within such markets.[9,10] In one study, virus isolation was made at even higher rates in poultry drinking water than in bird droppings randomly collected in the markets.[11]

The H5N1 highly pathogenic avian influenza (HPAI) virus is a major public health concern in Southeast Asia, where it has widely spread since its first detection in 1997.[12] Despite various prophylactic processes carried out in several countries, including poultry vaccination campaigns, the virus has become enzootic in the region. In Cambodia, since the first detection of the HPAI H5N1 virus in 2004, 18 human cases of infection (16 fatalities) and almost 30 outbreaks in poultry have been reported as of October 10th and 24th, 2011, respectively.[13,14]

The H5N1 virus has the ability to persist in different types of water,[15,16] and H5N1 viral RNA was previously detected in environmental specimens such as mud, pond water, aquatic plants and soil/dust swabs,[17–19] including within the surroundings of H5N1 outbreaks areas in Cambodia.[8]

In this country, human cases of H5N1 HPAI occurred mainly after direct contact with infected poultry,[20] although seroepidemiological studies identified bathing and swimming in ponds as other major risk factors for human contamination.[21,22] This is consistent with data reported from neighbouring countries, which also suggest that exposure to H5N1-contaminated environments (soiled water, poultry-slaughtering facilities, faeces-based fertilizer, litter)

without direct contact with infected poultry is associated with an increased risk of human infection.[23–27] The exact role of the environment in the transmission of H5N1 virus remains poorly understood. Few authors have described the survival of H5N1 virus in water, soil or various surfaces in laboratory-controlled conditions with temperatures usually ranging from 0 to 25°C,[15,16,28,29] but very little is known regarding the persistence of the virus in natural settings where outbreaks regularly occur, for example, in tropical countries where average temperatures can reach over 35°C in the shade. The purpose of this study was to investigate various environmental components as potential reservoirs for H5N1 virus and thus as potential sources for human and animal contamination.

Materials and methods

Sample collection

In response to the notification of confirmed cases of H5N1 infection in humans or poultry, we conducted four investigations in the households of the index cases and in the surrounding vicinities. Environmental specimens were collected in five households of three Cambodian provinces between April 2007 and February 2010 (Figure 1). These samples included water – collected in sterile tubes and containers – mud, aquatic plants and animals, poultry feathers, various domestic animal faeces and soil collected in sterile tubes, and dust swabs, moistened with viral transport medium (VTM) prior to collection and storage in VTM tubes afterwards. All specimens were kept at 4°C while being transferred to the laboratory within few hours and then stored at −80°C until testing.

BioSafety statement

All tests conducted on the samples were performed within the Bio-Safety level 3 Laboratory (BSL3) of Institut Pasteur in Cambodia.

Concentration of H5N1 virus in water

Two methods of influenza virus concentration in water were used. The first one was based on the biological property of the virus to agglutinate chicken red blood cells (CRBCs) as described previously[30] and was used to test small volumes of water (<50 ml). The second method consisted in an adsorption step on glass wool, followed by an elution step with a beef extract solution at alkaline pH, in combination with a final concentration step with polyethylene glycol (PEG), and was optimized for large volumes of water (up to 10 l).[31] The final concentrates obtained were used for nucleic acid extraction, HA5 haemagglutinin gene amplification and virus isolation.

Concentration of H5N1 virus from mud and soil specimens

Mud and soil specimens (5 g) were eluted with 25 ml of 10% beef extract solution at pH 7, followed by a PEG-pre-

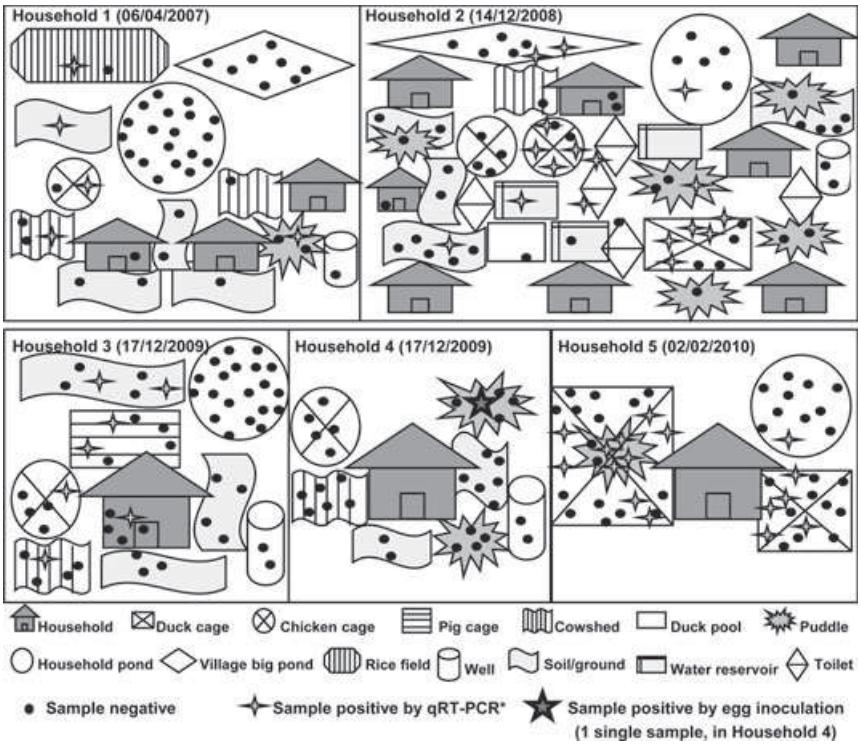

Figure 1. H5N1 virus detection in environmental specimens collected in five households. *A sample was considered as positive when it tested positive for HA5, M and NA1.

cipitation step for virus and RNA concentration.[32] The mud eluates and concentrates were then used for RNA extraction or inoculation into embryonated hen eggs for infectious viral particles isolation.

Homogenization of other solid samples (plants, straw, aquatic animals)

All aquatic animals (fish, snails, insects) and plants collected from ponds, as well as straw samples collected from poultry cages, went through an homogenization step using the MagNa Lyser Instrument (Roche Diagnostics, Mannheim, Germany) for three runs of 50 seconds at 5000 ***g***. Supernatants were then used for RNA and virus detection.

Total nucleic acid extraction and amplification by real-time RT-PCR

All samples processed as described above were mixed with an antibiotics solution (dilution 1/10) prior to further RNA extraction or virus isolation to reduce the number of contaminating organisms in the samples.[30,32] Either MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) with the MagNa Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany) or QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) was then used for viral RNA extractions on all eluted/concentrated/homogenized samples (200 μl), following the manufacturers' recommendations. Quantitative real-time RT-PCRs (qRT-PCRs) targeting the haemagglutinin (HA5), matrix (M) and neuraminidase (NA1) genes were performed on the extracted RNA, as described previously.[32] The qRT-PCR designed for HA5 detection was used to screen all the samples as it was the most sensitive of the three qRT-PCRs. The qRT-PCR targeting M gene was used only to confirm results on the specimens that tested positive for HA5, and the NA1 qRT-PCR was meant to confirm the virus subtype. A sample was declared positive for H5N1 virus when it tested positive with the three different qRT-PCRs.

Virus isolation in embryonated hen eggs

All samples that tested positive by qRT-PCR for all HA5, NA1 and M genes were subsequently inoculated into specific pathogen-free (SPF) embryonated hen eggs,[32] each sample being inoculated into three eggs. The eggs were then incubated for 48 hours at 37°C and chilled overnight at 4°C. Amnio-allantoic fluids (AAF) were harvested, and standard haemagglutination (HA) tests were performed to confirm the presence of virus. HA tests were performed in 96-well microtitre plates with 0·75% guinea pig red blood cells and serial twofold dilutions of AAF. Negative HA tests led to additional passages on eggs. A maximum of three passages were performed for each sample. Positive HA test was confirmed by HA5 qRT-PCR for virus identification.

Results

Between April 2007 and February 2010 during the investigation of four outbreaks of H5N1 virus in poultry, a total of 246 environmental specimens were collected in five households of three Cambodian provinces (Figure 1). Among these samples, 178 were collected from the farms' environment and 68 were collected exclusively from nearby ponds. Of the 246 samples collected, 46 (19%) tested positive by qRT-PCR targeting the HA5, NA1 and M genes (Table 1 and S1), out of which only one contained infectious H5N1 particles. At the time of investigation following the report of a human case, all poultry were already dead or the few surviving ducks already tested negative (data not shown).

H5N1 virus detection in specimens collected from farms' environment

H5N1 virus RNA was frequently detected in dust (including specimens collected inside the houses), soil and puddle mud samples obtained from the farms' environment (Table 1 and S1). These specimens often contained high numbers of RNA copies, with mean viral loads of $1{\cdot}2 \times 10^4$ RNA copies per ml of dust supernatant, $3{\cdot}1 \times 10^4$ RNA copies per gram of soil and $8{\cdot}9 \times 10^4$ RNA copies per gram of puddle mud. A third of the samples collected from duck cages tested positive by qRT-PCR. In particular, most of the few available duck feathers and straw specimens collected in the duck cages tested positive. As for the water samples, only two samples tested positive by qRT-PCR: one came from a puddle and the other was a sample of drinking water collected from a container used by ducks (Figure 1, Table 1 and S1). Overall, 42 of the 178 samples collected from within the farms' environment tested positive for H5N1 virus RNA. The highest viral loads were observed in contaminated straw ($4{\cdot}9 \times 10^5$ RNA copies per gram), puddle mud ($4{\cdot}5 \times 10^5$ RNA copies per gram) and duck drinking water (2×10^5 RNA copies per ml). Among these 42 specimens, there was only one for which virus isolation was successful. This infectious strain was isolated from a specimen of water collected from a puddle in household 4 (Figure 1). In this household, the last poultry death was reported 2 days prior to sampling date. The viral load measured by HA5 qRT-PCR in this water specimen was surprisingly low (10 copies per millilitre).

H5N1 virus detection in specimens collected from ponds

Of a total of 68 samples collected from ponds, four tested positive for H5N1 virus RNA.

Samples collected from ponds appeared to give a lower proportion of positive results by HA5 qRT-PCR (6%) as compared to samples collected elsewhere in the farms

Table 1. Influenza A H5N1 virus detection in environmental and animal specimens collected in five households*

Source	Sample type	Household identification*	No. of specimens tested positive by qRT-PCR (%)**	Total no. of samples tested positive by qRT-PCR (%)**	Viral load in copies of HA5 RNA per gram or per millilitre			No of samples positive by virus isolation
					Mean	Max.	Min.	
Farms	Dust	1	1/2 (50)	16/35 (46)	$1{\cdot}2 \times 10^4$	$8{\cdot}7 \times 10^4$	58	0
		2	6/14 (43)					
		3	3/4 (75)					
		4	0/5 (0)					
		5	6/10 (60)					
	Soil	1	2/9 (22)	12/81 (15)	$3{\cdot}1 \times 10^4$	3×10^5	68	0
		2	5/13 (38)					
		3	4/30 (13)					
		4	0/14 (0)					
		5	1/15 (6)					
	Mud	1	1/4 (25)	6/19 (31)	$8{\cdot}9 \times 10^4$	$4{\cdot}5 \times 10^5$	108	0
		2	1/5 (20)					
		4	0/6 (0)					
		5	4/4 (100)					
	Water (puddles, wells)	1	0/1 (0)	1/14 (7)	10	NA	NA	0
		2	0/6 (0)					0
		3	0/2 (0)					0
		4	1/5 (20)					1
	Drinking water collected inside duck cages	5	1/2 (50)	1/2 (50)	2×10^5	NA	NA	0
	Straw in duck cages	5	2/4 (50)	2/4 (50)	$2{\cdot}5 \times 10^4$	$4{\cdot}9 \times 10^5$	9000	0
	Duck feathers	1	1/1 (100)	2/2	320	473	167	0
		2	1/1 (100)	(100)				
	Duck tracheal swabs	2	1/4 (25)	1/4 (25)	147	NA	NA	0
	Duck faeces	2	0/5 (0)	1/10 (10)	$1{\cdot}7 \times 10^4$	NA	NA	0
		5	1/5 (20)					
	Domestic animal's faeces/rectal swab (dogs, bovines)	1	0/2 (0)	0/7 (0)	NA	NA	NA	0
		2	0/5 (0)					
Ponds	Mud	1	0/8 (0)	2/24 (8)	3050	5000	1100	0
		2	2/7 (28)					0
		3	0/3 (33)					0
		5	0/6 (0)					0
	Water	1	0/6 (0)	0/16 (0)	NA	NA	NA	0
		2	0/1 (0)					
		3	0/6 (0)					
		5	0/3 (0)					
	Aquatic plants	2	0/1 (0)	1/4 (25)	10^4	NA	NA	0
		3	0/1 (0)					
		5	1/2 (50)					
	Aquatic animals (fishes, shells, snails, insects, etc.)	1	0/13 (0)	1/24 (4)	2500	NA	NA	0
		2	1/4 (25)					
		3	0/5 (0)					
		5	0/2 (0)					
Total				46/246 (19)				1/246

NA, not applicable.

*Household 1: 6 April 2007 (date of investigation/sample collection), 11 April 2007 (date of last poultry death), 31·5°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 7·5 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 2: 14 December 2008 (date of investigation/sample collection), 7 December 2008 (date of last poultry death), 33°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 7·7 [pH of the water in pond (or in the well for household 4)], Kandal (province); household 3: 17 December 2009 (date of investigation/sample collection), 17 December 2009 (date of last poultry death), 31·7°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6·85 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 4: 17 December 2009 (date of investigation/sample collection), 15 December 2009 (date of last poultry death), 29·5°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6·1 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 5: 2 February 2010 (date of investigation/sample collection), 2 February 2010 (date of last poultry death), 34°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6·9 [pH of the water in pond (or in the well for household 4)], Takeo (province).

**A sample was declared positive when it tested positive with the three qRT-PCRs targeting the HA5, M and NA1 genes.

(24%). In ponds, H5N1 virus nucleic acid was detected in few mud samples (8%), in one aquatic plant sample and in one very small fish. All water specimens obtained from ponds tested negative by HA5 qRT-PCR. No infectious H5N1 particles were detected from any specimens collected from the ponds (Table 1).

Conclusions

Our investigation demonstrates that following H5N1 outbreaks in poultry, infectious particles and viral genome do persist in the environment of farms in Cambodia, even though infectious virus was successfully isolated from only one sample of water. Viral RNA was detected in 46% of dust swabs, 31% of mud specimens and 15% of soil samples collected from the farms' environment. These high proportions of positive specimens may be explained by the samples sources. Indeed, the majority of these specimens were collected in poultry cages or in cowshed and pig cages, which are usually located close to poultry cages where the virus is expected to be detected more frequently and in large amounts. The positivity rates obtained in our study were higher than the ones reported in Cambodia previously.[8] This may be explained by the testing method used. In our study, an elution and concentration step was performed on all mud and soil samples prior to viral RNA extraction, whereas Vong *et al.*[8] used direct extraction of nucleic acids without any pre-treatment of the environmental specimens, which was recently demonstrated to be less sensitive.

Avian influenza viruses were reported to have the ability to survive outside the host for a few days up to several months depending on the environmental conditions and viral concentrations.[15,16] However, our data suggest that in tropical countries, virus inactivation may occur rapidly due to several factors such as heat (temperature in ponds water ranging from 29·5 to 33°C and outside temperature exceeding sometimes 35°C during the investigations), salinity, dryness, ultraviolet radiation and pH.[16,33] Although the freeze/thaw step included in our collection and testing protocols should only be responsible for a small loss of virus titre, given the already very low viral load detected in some samples (Table 1), this step could partially explain why infectious particles were rarely recovered.

Although viral particles may not be infectious anymore, their RNA is still protected from degradation in the matrix protein and the core and could consequently be detected by qRT-PCR (HA5, NA1 and M genes).[3] As already described in a previous study,[32] PCR inhibitors were detected in almost 30% of the mud samples, but even after serial dilutions, these specimens still tested negative by HA5 qRT-PCR (data not shown). Unsurprisingly, viral loads detected in duck cages were among the highest. However, it is noteworthy that the one sample that tested positive for virus isolation had not been collected from a duck cage but from a puddle water sample, which also contained low quantities of viral RNA (10 RNA copies per ml). These data emphasize the idea that various physico-chemical or microbiological parameters may influence the survival of H5N1 viral particles in natural settings, in ways that are yet to be clarified.

The detection of influenza RNA in 8%, 25% and 4% of mud, aquatic plants and aquatic animals collected from ponds, respectively, should not be regarded as insignificant, especially as these ponds were located nearby (~100 m) the households of the index cases. These results suggest that aquatic sites should be considered as a potential source for human and/or animal infection. Indeed, animals are drinking this water and ducks are swimming in ponds and can therefore contaminate the aquatic environment but also be contaminated. In addition, the ponds are also commonly used by children for playing and swimming, and this behaviour was identified as a risk factor for subclinical human contamination.[21,22]

The duration of survival of the virus was estimated through the interval of time between the last poultry death and the sample collection. Here, in environmental samples collected 7 days after the last bird's death, virus RNA was still detected, even though infectious virus could not be isolated in eggs. This was in agreement with our previous findings.[8] This work supports the idea that environmental sampling is a valuable approach to assess the presence and evaluate the extent of the dissemination of influenza viruses in specific geographical or environmental locations.[7] Our findings also demonstrate that during H5N1 virus outbreaks in tropical areas, many environmental components surrounding outbreak areas are widely contaminated by the virus and may act, probably for only a short period of time (during or just after the virus is shed by poultry), as potential sources for human and/or animal contamination as already suggested several times over the last few years.[21–27] For instance, our data showing a relatively high H5N1 virus detection rate in dust samples raise concerns about a possible airway transmission by inhalation of infectious particles in suspension in the air. In addition, previous studies demonstrated the possibility of H5N1 infection through oral route in mammals,[34] along with the description of an intestinal syndrome in some human cases.[20,23] This supports the hypothesis that inhalation of infected droplets through direct contact with infected poultry might not be the only possible way of human contamination. Thus, our results underscore the importance for regular surveillance and disinfection of the farms' environment following avian influenza outbreaks.

Further investigations in outbreak areas and around live bird markets should be carried out to complete the current

pool of data available on the persistence of HPAI H5N1 virus in natural environment in endemic tropical countries. Indeed, understanding the complete epidemiology of H5N1 virus is important for the prevention of human, wildlife and domestic animal disease caused by this virus. Now that better diagnostic methods have been described for H5N1 detection in water and mud samples.[31,32] and surveillance of H5N1 virus in the environment could be an interesting tool to monitor virus circulation and risk of exposure for humans and animals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Distribution by sample source of the specimens which tested positive by qRT-PCR for HA5, NA1 and M genes.

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4. Conclusions de l'étude

La présence d'ARN viral du virus H5N1 été détectée dans 19% des échantillons environnementaux. Cette détection a lieu le plus fréquemment dans des écouvillons de poussière, de boue, et de sol (46, 31 et 15 % respectivement) prélevés dans l'environnement immédiat des fermes où des foyers épidémiques ont été déclarés. Les échantillons collectés dans les mares présentent des niveaux de détection d'ARN viral plus bas (6%) que ceux collectés dans les fermes (24%). Des particules virales infectieuses n'ont été isolées que dans un seul échantillon d'eau prélevé dans la vase d'une flaque d'eau.

Cette étude montre qu'au cours des épizooties à virus , de nombreux éléments de l'environnement entourant les zones d'épidémies sont contaminés par le virus et peuvent agir comme des sources potentielles de contamination humaine et /ou animale. En complément de cette étude et pour mieux comprendre la persistance du virus H5N1 dans l'environnement aquatique, une étude expérimentale a été menée en conditions reproduisant le plus fidèlement possible le biotope de mares ou de lacs au Cambodge (Chapitre 4).

CHAPITRE 4

Etude de la survie du virus influenza H5N1 dans des environnements aquatiques artificiels.

1. Contexte de l'étude

Il existe peu d'information sur la persistance du virus H5N1 dans l'environnement en zone tropicale bien qu'il ait été montré que l'environnement pouvait jouer un rôle de réservoir et constituer une source potentielle de contamination humaine et / ou animale (Breban et al., 2009; Iglesias et al., 2010; Van Kerkhove et al., 2011; Vong et al., 2008; Vong et al., 2009; WHO, 2007). Le virus H5N1 peut persister hors de l'hôte dans l'eau (Domanska-Blicharz et al., 2010; Iglesias et al., 2010; Lebarbenchon et al., 2010; Stallknecht and Brown, 2009; Van Kerkhove et al., 2011) et l'ARN viral peut être détecté dans l'environnement (Vong et al., 2008). Bien que la survie du virus H5N1 ait été démontrée dans l'eau, le sol et différentes surfaces dans des conditions contrôlées en laboratoire, avec des températures généralement comprises entre 0 et 25 °C (Brown et al., 2007a; Brown et al., 2007b; Domanska-Blicharz et al., 2010; Paek et al., 2010; Stallknecht and Brown, 2009), très peu de choses sont connues quant à la survie du virus dans les conditions réelles d'un milieu tropical. De même, peu de choses sont connues sur le rôle potentiel des animaux aquatiques comme réservoirs et intervenants dans la transmission du virus dans l'eau et à d'autres animaux aquatiques.

2. Objectifs de l'étude

Notre objectif dans cette étude est:

1) Décrire la survie du virus H5N1 dans l'eau et la boue placées dans un montage expérimental reproduisant le plus possible l'état naturel observé dans les pays tropicaux;

2) Observer si les animaux aquatiques comme les poissons, les têtards, les coquillages, les escargots et la flore aquatiques peuvent être contaminés et jouer un rôle dans la persistance du virus H5N1 dans l'eau;

3) Observer si les poissons et les moules pouvaient jouer un rôle dans la transmission du virus H5N1.

3. Résultat de l'étude

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Influenza A(H5N1) survival in complex artificial aquatic biotopes

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Running title: Influenza A(H5N1) survival in water

Keywords: H5N1 virus, survival, RNA persistence, water, aquatic animals

Abstract

Background: Very little is known regarding the persistence of Highly Pathogenic Avian Influenza (HPAI) H5N1 viruses in aquatic environments in tropical countries, although environmental materials have been suggested to play a role as reservoirs and sources of transmission for H5N1 viruses.

Methodology/Principal Findings: The survival of HPAI H5N1 viruses in experimental aquatic biotopes (water, mud, aquatic flora and fauna) relevant to field conditions in Cambodia were investigated. Artificial aquatic biotopes, including simple ones containing only mud and water, and complex biotopes involving the presence of aquatic flora and fauna, were set up. They were experimentally contaminated with H5N1 virus. The persistence of HPAI H5N1 virus (local avian and human isolates) was determined by virus isolation in embryonated chicken eggs and by real-time reverse-polymerase chain reaction. Persistence of infectious virus did not exceed 4 days, and was only identified in rain water. No infectious virus particles were detected in pond and lake water or mud even when high inoculum doses were used. However, viral RNA persisted up to 20 days in rain water and 7 days in pond or lake water. Viral RNA was also detected in mud samples, up to 14 days post-contamination in several cases. Infectious virus and viral RNA was detected in few cases in the aquatic fauna and flora, especially in bivalves and labyrinth fish, although these organisms seemed to be mostly passive carriers of the virus rather than host allowing virus replication.

Conclusions/Significance: Although several factors for the survival and persistence of HPAI viruses in the environment are still to be elucidated, and are particularly hard to control in laboratory conditions, our results, along with previous data, support the idea that environmental surveillance is of major relevance for avian influenza control programs.

Introduction

The highly pathogenic avian influenza (HPAI) H5N1 virus is a major public health concern in Southeast Asia, where it has widely spread since its first detection in 1997 [1] and become enzootic in the region. Cambodia is one of the enzootic countries in tropical areas which has reported a high fatality rate in humans (approximately 90%) [2]. Since the first detection of HPAI H5N1 virus in poultry in 2004 and the first human cases of H5N1 virus infection in 2005, 32 H5N1 outbreaks in poultry and 18 human cases (16 fatalities) of H5N1 infection have occurred up to now [2,3]. Direct contact with infected poultry is the main source for human contamination. However, previous studies provide additional evidence suggesting bathing or swimming in ponds as a risk factor for human H5N1 contamination [4]. The H5N1 virus has been shown to have the ability to persist outside the host, especially in water [5-9] and H5N1 viral RNA was previously detected in environmental specimens, including in the surroundings of H5N1 outbreaks areas in Cambodia [10]. Previous studies have described the survival of H5N1 virus in water, soil or various surfaces in laboratory-controlled conditions with temperatures usually ranging from 0 to 25°C [5,8,11,12] but very little is known regarding the persistence of the virus in environment materials such as surface water, mud, soil in tropical countries where average temperatures can reach over 35°C in the shade. Data on the ability of HPAI H5N1 viruses to remain infective outside of the host is very limited. There also are very few reports discussing the role of aquatic fauna in the transmission cycle of the H5N1 virus. An experimental study conducted with low pathogenic avian influenza (LPAI) demonstrated that Asian clams (*Corbicula fluminea*) were capable of removing and reducing the infectivity of avian influenza viruses (AIVs) in water [13]. On the other hand, a study by Stumpf et al. showed that zebra mussels (*Dreissena polymorpha*) were able to accumulate LPAI virus from the surrounding water and to retain the virus in their bodies over an extended period of time before releasing the virus back into freshwater [14]. These few

studies seem to emphasize the need for more relevant data on the survival of HPAI H5N1 virus in natural aquatic environments, including in the presence of aquatic fauna.

Our objectives in this study were: (1) to describe the survival of H5N1 virus in water and mud in experimental setting reproducing as faithfully as possible natural conditions observed in tropical countries; (2) to determine whether aquatic animals such as fish, tadpoles, clams, snails, mussels and aquatic flora may be contaminated and play a role in the persistence of H5N1 virus in water; (3) to determine whether autochthonous aquatic organisms such as bivalves (fresh water mussels) and labyrinth fish (fighting fish) could transmit the virus to each other.

Materials and methods

BioSafety statement

All experiments using HPAI H5N1 virus and all animal experiments were performed within the Biosafety Level 3 (BSL-3) laboratory of the Institut Pasteur in Cambodia (IPC), complying with the Animal Committee regulations of Institut Pasteur in Paris, France, in accordance with the EC 86/609/CEE directive, and approved by the Animal Ethics Committee of Institut Pasteur in Cambodia (permit number: AEC/IPC/002/2008).

Virus

The clade 1, genotype Z, HPAI H5N1 viruses A/Cambodia/408008/2005 (GenBank accession numbers: HQ664938 to HQ664945) and A/chicken/Cambodia/LC1AL/2007 (GenBank accession numbers: HQ200574 to HQ200581) were used to conduct these experiments. The virus stock was obtained after propagation in Specific Pathogen Free (SPF) 9-to-11-day-old embryonated hen eggs, kindly provided by the National Veterinary Research Institute of Cambodia (NaVRI), Ministry of Agriculture, Forestries and Fisheries (MAFF). The amnio-allantoic fluid (AAF) from the second passage on SPF eggs was harvested 48

hours after inoculation and stored at -80°C until further use. Virus titre was determined by calculating the 50% egg infectious dose (EID50) per mL of virus stock. Titration endpoints were calculated using the method of Reed and Muench [15].

Mud and water

Mud and water used in the artificial aquatic settings described below were collected from 2 different ponds (with the landlord's official authorization) and from a lake in areas of Kampong Cham province where an H5N1 virus outbreak had previously occurred. Some of the experiments also involved the use of rain water which was collected and stored in big jars within the IPC external facilities until use. Temperature, pH and conductivity results of all water samples were recorded on site at the time of collection. Additional physico-chemical analyses and all microbiological tests were carried out upon arrival at the laboratory (Supplementary Table 1). All water and mud samples were transported at ambient temperature (~30-35°C) from the collection site to the laboratory within 5 hours. These samples were used to conduct the experiments within 24 hours after field collection. In the meantime, they were kept at room temperature (~20-25°C). The absence of virus in all water and mud samples was verified by qRT-PCR prior to use for the experiments. Microbiological and additional physico-chemical parameters were measured in the water samples at the beginning of the experiments; pH and conductivity results demonstrated very few or no differences with the measures made previously in the field (data not shown).

Aquatic flora and fauna

Freshwater flora and fauna used for the artificial aquatic settings included guppies (*Poecilia reticulata*), Siamese fighting fish (*Betta splendens*), tadpoles (unidentified local species), snails *(Sinotaia quadrata)*, clams (*Corbicula fluminea*), mussels *(Pilsbryoconcha exilis)*, and aquatic plants (*Cabomba caroliniana*). Fish were bought from a private stockbreeder and the

other organisms were collected from local rivers or ponds where H5N1 virus circulation was never reported (no permits required).

Artificial aquatic biotopes: experimental design and settings

A total of 4 different series of experiments were carried out. Two series were conducted to investigate the survival and persistence of H5N1 virus in simple biotopes containing only water and mud (A), and in complex biotopes that included aquatic flora and fauna (B) (See Table 1 for details). Two additional series of experiments were set up in order to precisely characterize the role of some aquatic animals in the persistence of H5N1 virus in aquatic environments. The roles of mussels (bivalve molluscs) (C) and endemic *Betta splendens* fish (fighting fish; *Osphronemidae* family) as well as tadpoles (D) were investigated (see Figures 1 and 2 for details).

Different virus concentrations were tested in this study. Concentrations of 2 10^2 and 5 10^2 EID50/mL of water were chosen based on the quantity of virus found in the natural environment in Cambodia during previous field studies [10,16]. The virus concentration of 5 10^3 EID50/mL of water was determined based on the estimation of the quantity of virus particles that infected ducks might shed in a pond (number of ducks adjusted to the size of the pond according to field observations) [17]. Finally, a higher dose of virus (5 10^4 EID50/mL of water) was also tested in order to study the virus persistence in case of higher level of contamination.

Experiments of series A and B lasted 14 days each, and were conducted using different H5N1 strains (A/Chicken/Cambodia/LC1AL/2007 and A/Cambodia/408008/2005), different inoculum doses (yielding final estimated concentrations of 5 10^2, 5 10^3 or 5 10^4 EID50/mL water), and different temperatures reflecting the parameters measured in the field during the transmission season in Cambodia (22, 25, 32 or 34°C). Aquariums with a total capacity of 28 litres (38 38 20cm) were filled with 20 litres water and 5 kilograms mud

144 each, and then were allowed to settle for 24 hours prior to virus inoculation. Water and mud
145 from various origins were also tested: rain, lake, pond 1, pond 2 (as defined above). When
146 flora (about 100 g) and fauna (30 animals of each species) were included in the experiments
147 (B), collection of samples from each of the species used (2 g of plant, 2 animals of each
148 species) was carried out during the first 3 days, and every 3 days from then on. Fifty
149 millilitres of water and 5 grams of mud samples were collected on a daily basis (see Table 1
150 for details.
151 Experiments in series C and D lasted from 8 to 20 days depending on the setting chosen. The
152 A/Cambodia/408008/2005 strain was used for experiments C, while experiments D involved
153 the use of the virus A/Chicken/Cambodia/LC1AL/2007. All biotopes created for experiments
154 C and D used rain water only. Aquariums filled with 10 litres of water and 40 mussels each
155 were used for experiments C. In experiments D, series of small aquariums containing 500 mL
156 of water and one male fighting fish in addition to one tadpole were used for experiment D1,
157 while only one fighting fish was included in experiment D2 (Figure 2). In experiments C, the
158 water was maintained at 25°C at all times, while it was kept at ambient BLS-3 laboratory
159 temperatures for experiments D. The temperature measured in the water during experiment D
160 varied from 15.1 to 22.5°C, but usually stayed around 18-20°C with an average temperature
161 of 17.4°C (Supplementary Figure 1). Final estimated virus concentrations in aquariums varied
162 between 2 10^2 (D) and 5 10^4 EID50/mL water (C). Water samples of 50 and 10 mL were
163 collected daily during experiments C and D, respectively (Figures 1 and 2).
164 For all aquariums, inoculation was carried out on day 0 (D0). After collection, water and mud
165 samples were stored in sterile tubes at -80°C until testing. All aquatic animals were humanely
166 sacrificed, following the Animal Use Protocol defined by the Animal Ethics Committee of
167 Institut Pasteur in Cambodia (permit number: AEC/IPC/004/2008), and subsequently
168 dissected in order to collect the main organs of interest: gills, intestines, fins, scales, brain,

169 and remaining carcass in fish; gills, digestive gland, intestines, and remaining carcass in clams
170 and mussels; gills, intestines, and remaining carcass in tadpoles; all organs in snails. Before
171 dissection, the animals were washed 2 times with sterile distilled water in order to avoid
172 contamination of the organs by the water contained in the aquarium.
173 Prior to testing, organs were weighed and placed into vials containing 1 mL of viral transport
174 medium (VTM) (sterile solution at pH 7.2-7.4 containing 26.5g/l of tryptose phosphate broth,
175 5g/l of gelatine, 50mg/l of fungizon, 1 million units/l of penicillin, 1 g/l of streptomycin and
176 80mg/l of gentamycin) and stored at -80°C and in 10% formalin solution (prepared from
177 formaldehyde 37% commercial solution diluted in water) stored at room temperature. Plant
178 samples were also weighed and stored in VTM at -80°C until use.
179 Each experiment conducted was coupled with a control experiment, using the exactly same
180 conditions, but without virus.
181
182 ***Preparation of samples for RNA extraction and/or virus isolation***
183 ***Virus concentration in water***
184 All water samples were concentrated using the method described by Khalenkov et al. [18], to
185 obtain a final volume of 1 mL of concentrate for each sample. The limit of detection of this
186 technique was 3×10^{-2} EID50/mL, as previously determined [18].
187 ***Virus elution and concentration in mud***
188 All mud specimens collected went through an elution step with a 10% beef extract solution at
189 pH 7 followed by a polyethylene glycol-precipitation (PEG) step for virus and RNA
190 concentration, as described previously [19]. The limit of detection of H5N1 virus in mud was
191 1.6 10^4 RNA copies/g of mud (approximately 50 TCID50/g of mud) [20].
192 Precisely, for each mud sample, 5 grams were eluted in 25 mL of elution buffer (10% beef
193 extract solution). One millilitre of the eluted sample was then kept for a first virus detection,

while the remaining volume went through the additional concentration step with PEG so as to obtain a final volume of concentrated sample of 1 mL. Mud eluates and concentrates were then used for RNA extraction and qRT-PCR or virus isolation.

Homogenization of animal and vegetal samples

All solid samples (animals' organs, plants) were weighed and kept in 1 mL VTM before undergoing a homogenization step using the MagNa Lyser Instrument (ROCHE, Mannheim, Germany) for 3 runs of 50 seconds at 5000 g. The homogenized samples were then used for further RNA extraction and eventually virus isolation.

Total nucleic acid extraction and amplification by real-time RT-PCR

All samples processed as described above were mixed with a solution containing a mixture of antibiotics and antifungal drugs prior to RNA extraction or virus isolation, in order to reduce the number of contaminating microorganisms in the samples [18,19]. MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) on MagNa Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany) were then used for viral RNA extraction of all eluated/concentrated/homogenized samples (200µL), following the manufacturers' recommendations. Quantitative real-time RT-PCR (qRT-PCR) targeting the hemagglutinin (H5), matrix (MA) and neuraminidase (N1) genes were performed on all RNA extracted. H5, MA and N1 synthetic RNA were used as internal controls and for quantification. Water and mud samples mixed with H5N1 virus at a concentration higher than the limit of detection were used as positive controls [19]. No positive controls were available for testing the animal and plant specimens.

Virus isolation in embryonated hen eggs

All samples that tested positive by qRT-PCR were inoculated into 9-to-11-days old SPF embryonated hen eggs. Each specimen was inoculated into 3 eggs. One hundred microlitres were injected into the amniotic cavity and 100 µL into the allantoic cavity. The eggs were

then incubated for 48 hours at 37°C and chilled overnight at 4°C. The AAF was then harvested and standard hemagglutination (HA) tests were performed to detect the presence of virus before confirmation by qRT-PCR. HA tests were performed in 96-wells microtiter plates with 0.75% guinea pig red blood cells and serial 2-fold dilutions of AAF. When the HA test was negative, the AAF from each of the three eggs was pooled and inoculated into a second and then a third series of 3 eggs. A maximum of three passages were performed for each sample.

Histopathology and immunohistochemistry

Immunohistochemical staining of the tissues obtained from the mussels and fish of experiment C and D was carried out for the influenza nucleoprotein using HB65 (European Veterinary Laboratories, Netherlands) as described in previously published reports [21].

Results

Survival of infectious particles and persistence of virus RNA in water

Contaminated rain water was the only type of water from which infectious particles could be recovered (Figures 3 and 5; Supplementary Table 2), although these viruses could not be detected any later than 4 days post-inoculation. In the same experimental conditions, the virus of human origin could not be isolated in embryonated eggs and a trace of its RNA was detected for a shorter period of time than when using the avian isolate (experiment A.1, Figure 3; Supplementary Table 2). When animals (without plants or mud) were introduced (experiments C with mussels and D with fish and tadpoles) (Figure 5; Supplementary Table 2), infectious particles of both animal and human origins were then isolated and the RNA persisted for a longer period than in the presence of mud and plants, and at higher levels, especially when the average water temperature was low (17°C).

No infectious particle could be isolated from contaminated lake water, although viral RNA was detectable between 1 to 11 days post-inoculation depending on the conditions tested (Figures 3 and 4; Supplementary Table 2).

No infectious particles could be recovered from pond water. In one instance, viral RNA persisted in water for as long as 14 days (end of the experiment) (experiment A.2.2, Pond 1, Figure 3; Supplementary Table 2) although at very low titre at this last testing point (52 RNA copies/mL). In all other experiments, viral RNA was detected from contaminated pond water, from 2 to 14 days post-inoculation, with viral loads varying from 2 to 1700 RNA copies/mL.

Survival of infectious particles and persistence of virus RNA in mud

Infectious particles could not be recovered from any mud samples in any of the experiments conducted in this study (Figure 3 and 4; Supplementary Table 3). Viral RNA could always be detected by qRT-PCR in mud specimens between 1 to 14 days after inoculation. When only considering the mud samples obtained from the lake, the RNA of the avian strain seemed to persist for longer periods (13-14 days) than the RNA of the human isolate (1-8 days), regardless of the other parameters (experiment B.1, Figure 4; Supplementary Tables 3 and 7). Globally, in experiments A and B using the avian strain, RNA was detectable for longer periods of time in mud (10 to 14 days) than in water specimens (1 to 7 days), except for one experiment in which viral RNA was still detectable in both water and mud specimens until the very end of the experiment (experiment A.2.2, Figure 3 and 4; Supplementary Tables 2, 3 and 7). The viral loads measured in the water samples were on an average 3000 times lower than those observed in mud specimens. With the human strain the durations of RNA persistence in water and mud were comparable but the viral loads were approximately 4700 times higher in mud than in water (Supplementary Table 7).

Survival of infectious particles and persistence of virus RNA in aquatic flora and fauna

Infectious particles were only isolated from animal organs in experiments C and D, in which mussels, tadpoles and fighting fish were immersed in contaminated rain water in the absence of mud (Figure 5, Supplementary Table 4). Survival of infectious particles ranged from 1 day in tadpoles and fighting fish (H5N1 strain of avian origin) to 6 days in mussels (with virus of human origin). RNA was detected in tadpoles up to 14 days after immersion in contaminated water (last day of the experiment) and until the end of respectively the 8 and 20 days of experiment in mussel and fighting fish. Viral loads detected in the organs of these animals were relatively high, ranging from 10^2 to 10^7 copies per gram (Figure 1 and 2; Supplementary Tables 4-6). The viral loads detected in the different organs of mussels and fish tested did not show any significant tendencies, and thus did not allow us to draw any conclusions regarding possible specific H5N1 tropisms towards certain organs in these aquatic animals, be it for the avian or the human strain (Supplementary Tables 5 and 6). As for the other aquatic animals and for the plants, only few samples tested positive by RT-PCR (Figure 4; Supplementary Table 4). Viral RNA persistence varied from 1 to 9 days. RNA could not be detected in the snail species used, nor in any of the animals or plants maintained at a temperature > 32°C (Figure 4; Supplementary Table 4). RNA from the human strain persisted only in guppy fish, whereas RNA from H5N1 virus of avian origin was detected only in clams.

The clams immersed in contaminated water died very quickly, in contrast with those immersed in non-infected water. The presence of mud in lake or pond water (experiments B.1 and B.2) was associated with the absence of infectious particle isolation in tadpoles and with a shorter persistence of virus RNA at a lower viral load (Figure 4; Supplementary Table 4). In contrast, in the absence of mud, infectious virus was detected on day 1 and the virus RNA persisted for at least 14 days at a high viral load in the animals (experiment D, Figure 5; Supplementary Tables 4,6 and 7).

Transmission of H5N1 virus between bivalves mollucs (experiments C) and between fighting fish (experiments D)

After immersion of mussels in rain water maintained at 25°C and contaminated with the virus A/Cambodia/408008/2005, infectious particles were isolated from water until day 4, from mussels until day 6 and the virus RNA was still detectable in molluscs until the end of the experiment at day 8. Viral loads in mussels varied between 2.40 10^5 copies on day 1 and 2.30 10^4 copies per gram of organ on day 8 (experiment C.2, Figure 1).

When transferred into non-contaminated water on day 3 (experiment C.1), RNA was detected in water until day 4 but infectious particles were not found. Infectious virus was not isolated in the infected mussels transferred into clean water on day 6 but the qRT-PCR tested positive until the end of the experiment (experiment C.1., Figure 1). The last viral loads measured were comparable to those of the mussels of the group maintained in contaminated water and interestingly, comparable quantities of RNA were detected in contaminated and exposed molluscs (experiment C.1, Figure 1; Supplementary Table 5).

When infected mussels were introduced into clean water on day 4 (experiment C.2), no virus could be isolated from the molluscs on day 5, and the water was not contaminated by infectious particles nor by RNA. However, RNA was still detected in infected mussels until day 8 (end of the experiments) and until day 7 in mussels exposed to infected ones (experiment C.2, Figure 1; Supplementary Table 5).

During the experiment D, fighting fish and tadpoles were immersed in contaminated rain water. The virus was isolated from animal organs until day 1 in both species and in water until day 2. RNA was detected in tadpoles until day 14 (last tadpole tested) and until day 20 (end of the experiment) in both water and fish. Viral loads varied from 2.8 10^7 copies on day 1 to 1.55 10^5 copies per gram of organ on day 14 for tadpoles, and from 4.73 10^6 copies to 1.48 10^4 copies per gram of organ on day 20 for fighting fish (experiment D.1, Figure 2).

316 When contaminated fighting fish were placed into clean water on day 6, no infectious virus
317 could be isolated from fish or from water. RNA persisted in water until the end of the
318 experiment on day 12 (viral load : 1.60 10^2 copies/mL) but in fish only until D7, in both
319 infected and exposed animals with 1 log difference in viral loads measured in their organs
320 (experiment D.2, Figure 2).
321 The viral loads presented here referred to the highest individual values found when analyzing
322 the different organs in each animal group (Supplementary Tables 5 and 6). In guppy fish, the
323 highest mean viral load was measured in gills (9.1 10^5 copies/g) followed by fins (4.8 10^5
324 copies/g), while the values obtained in the other organs varied from 4.5 10^4 to 2.8 10^5
325 copies/g. In contaminated fighting fish, the highest mean viral loads were observed in gills
326 (1.05 10^6 copies/g) and brain (4.11 10^5 copies/g), while the viral loads measured in the
327 other organs collected varied from 1.90 10^3 to 2.93 10^6 copies/g (Supplementary Table 6).
328 In tadpoles, the viral loads were quite similar in all organs (between 1.79 and 2.80 10^7
329 copies/g for tadpole immersed during 1 day in infected water and between 9.83 x10^4 and
330 5.82 10^5 copies/g for tadpole kept 13-14 days in contaminated water) (Supplementary Table
331 6). In clams, viral loads varied between 3.8 10^3 and 6.5 10^4 copies/g depending on the
332 organ tested.
333 ***Histopathology and immunohistochemistry***
334 Immunohistochemical staining of the tissues did not confirm the presence of the H5N1 virus
335 antigen in any organ of the mussels and fish tested following experiments C and D.
336
337 **Discussion**
338 This study aimed to recreate simple as well as complex aquatic environments with parameters
339 (pH, temperature, salinity, microorganisms, flora, fauna, etc.) as close as possible to those

observed in Cambodia, where H5N1 outbreaks are regularly reported, and to observe the survival of the HPAI H5N1 virus in all the different compartments of these artificial aquatic biotopes which have been suggested to be at the origin of asymptomatic or sub-clinical human infections [4,22]. Infected ducks can shed a large number of virus particles in their faeces but also in saliva and nasal discharge which can therefore easily lead to water contamination [23]. The survival of avian influenza viruses in natural or artificial environments has already been studied in several occasions and a recent review of Stallknecht and Brown commented that the persistence of HPAI H5N1 virus in the environment was still poorly explored [8].

In our experiments, infectious HPAI H5N1 virus could be recovered from water during a maximum of 4 days post-contamination at 25°C but only in rain water. This temperature is commonly observed all year long in Cambodian ponds and lakes, around 20-40 cm beneath the surface, as opposed to the surface where the temperature can easily exceed 30°C. The survival of AIVs in water is known to be shorter when temperature increases [8]. Interestingly, in similar conditions, infectious particles could not be isolated from any of the natural surface water specimens tested (ponds and lake). The pH values measured in this study varied between 7.45 and 8 which were described to be the optimal conditions to maintain the AIVs infectivity [8]. The main physicochemical and microbiological parameters which differed between rain and pond/lake water specimens were: a total absence of chemical oxygen demand (parameter used to indirectly evaluate the organic compounds) with globally lower concentrations of nitrite and nitrate, a higher concentration of sodium and a globally less abundant bacteriological flora in rain water (Supplementary Table 1). Nazir et al. examined the survival of low pathogenic avian influenza (LPAI) strains and reported that at 20-30°C, the persistence of the viruses was longest in distilled water, second longest in normal saline solution and shortest in surface water [24]. Others demonstrated that the presence of living microorganisms in some waters reduced AIV survival [5,23-25]. These

365 data are in line with our observations which suggest that at the temperature naturally observed
366 in tropical countries like Cambodia, the presence of organic contaminants and
367 microorganisms in natural surface waters are strongly affecting the H5N1 virus survival in
368 water. Additionally, although our samples underwent standard bacteriological analyses, water
369 specimens could have contained a whole range of other microorganisms, including fungi and
370 other microbes, which have not been investigated and which could have potentially been
371 interacting in some unknown way with influenza virus particles. Clean water, which can be
372 found in wells, in some containers, in puddles, etc., are in contrast favourable to the H5N1
373 virus survival and this seems not to be depending on the initial concentration of the virus i.e.
374 the level of virus contamination.
375 Interestingly, although experiments were conducted under identical conditions, the H5N1
376 virus obtained from a human case did not survive in rain water in the absence of fauna. Other
377 authors reported that in experiments where only pH, salinity and temperatures varied, H5N1
378 viruses appeared to persist for shorter periods than other avian influenza viruses tested [11].
379 This demonstrated an inter-subtype variation of virus tenacity in water but our results also
380 suggest the existence of an important intra-subtype variation that could be explained by
381 biological variations resulting from differing replication abilities in different hosts, or by yet
382 unknown genetic mutations associated with virus survival in abiotic environments.
383 The detection of virus RNA by qRT-PCR did not correlate with the recovery of infectious
384 particles. Indeed, in some experiments, infectious virus could not be isolated while RNA
385 could be detected for several days. In the absence of mud, plants or animals, RNA was
386 detected for periods as long as 11 days at 25°C. In complex biotopes, an increase of the
387 temperature from 25°C to 32°C or 34°C reduced the persistence of the RNA (experiments
388 A2.2, Figure 3; Supplementary Table 2). This is not surprising as RNA is known to be heat
389 labile

Infectious particles were never isolated in mud specimens although the method used was proven to be efficient [19]. LPAI viruses were reported to survive between 2 and 4 days at temperatures ranging from 20 to 30°C in some lake sediments [26]. The nature of the soil in Cambodia or the biological characteristics of the HPAI H5N1 virus may explain why the viruses did not survive in our mud specimens. It has been described that avian influenza viruses are relatively unstable in the environment due to their lipid envelopes readily being inactivated by several physical factors, organic solvents, and detergents [23]. However, this low detection of infectious particles may also be related to detection limits. For instance, adsorption of live virus on soil micro-particles, or contamination of the samples with environmental bacteria, fungi, or other microorganisms despite prior treatment, could prevent the growth of the virus in hen egg cultures [18,27-30]. As in water, the persistence of the avian H5N1 RNA tends to last longer than that of the human H5N1 strain in the mud, possibly for the same reasons as suggested above. Moreover, RNA persisted for longer periods in mud than in water. Previous publications supported the idea that AIVs could survive for longer in lake sediments than in lake water [26] and that lake and pond sediments could act as a reservoir of influenza viruses [31]. Our experiments cannot lead to similar conclusions as we did not isolate infectious particles from mud but mud and sediments may be preventing RNA from decay within the nucleoprotein, thus allowing it to be detected by qRT-PCR [31-33] even though PCR inhibitors are expected to decrease the detection rate of viral RNA in mud. Indeed, in our experiments, such inhibitors were detected in 50% of the soil and mud samples collected from the natural environment in Cambodia (Institut Pasteur in Cambodia, unpublished data). Several authors demonstrated that virus detection in environmental samples could indeed be strongly influenced by many substances present in environmental samples, such as bentonite clay, humic acid or mussel tissue, that can inhibit RT-PCR [34].

The detection thresholds of the assays could also be questioned but the quantity of virus inoculated at the beginning of the experiments should have ended in theoretical concentrations in water and mud above the limit of detection of these methods. Nevertheless, as we did not perform back-titrations immediately after virus inoculation in water, we cannot dismiss the possibility that the starting concentrations were lower than those calculated by simply applying a dilution factor. The initial virus titers used may appear low but they were comparable to those observed in the field during environmental investigation following outbreak in poultry in Cambodian farms [10,16]. Indeed, one of the main objective of these experiments was to study the persistence of H5N1 virus in conditions as close as possible to the field.The low virus isolation rate could be partially explained by a non-uniform distribution of the virus in the aquarium, although we tried to limit this bias by gently homogenizing the water in the aquarium with a long pipette and by collecting each sample at 4-5 different locations.

To our knowledge, data related to the infection of aquatic animals by AIVs in general is very rare and we did not find any study evaluating interactions between H5N1 virus and aquatic plants either.

The plants maintained in H5N1- infected water in conditions meant to simulate natural ones in Cambodia did not show any contamination by the virus regardless of the virus type and of the virus concentration except for one plant specimen in which viral RNA was detected during the first day of the experiment. This finding is in agreement with the investigation conducted on natural environmental samples collected after an H5N1 outbreak in Cambodia in 2006, which assessed the presence of viral RNA in plants from which no live virus particles were recovered [10]. This suggests that aquatic plants may not help virus survival, nor act as physical support for viral particles dispersed in water.

When molluscs, fish or tadpoles were introduced in aquariums containing rain water, infectious particles of the strain of human origin could subsequently be recovered and the RNA persistence of both human and avian strains increased significantly (up to 20 days in one experiment). This suggests a probable impact of these aquatic animals on the biological cycle of H5N1 virus. Faust et al. highlighted in 2009 the role of clams (*Corbicula fluminea*) in removing - by filtration - the virus from the water, and in reducing the infectivity of LPAI virus [13]. Another study reported that zebra mussels (*Dreissena polymorpha*) were able to accumulate LPAI virus from the surrounding water, and to retain the virus in their bodies over an extended period of time before releasing the virus back into freshwater [14]. As shown on Figure 1, the viral RNA persisted in water until the last day of the experiment (day 8) even in the presence of mussels. We observed a decrease of the viral load measured in water (3 logs in 8 days) but also in mussels (1 log in 8 day). In experiment C, infectious particles were detected in water during 4 days, and during 6 days in mussels (Figure 1). Once transferred to clean water, the infectious particles disappeared from the mussels and did not contaminate the water. Virus RNA was detected for few days in infected and exposed mussels. It seems that the species of mussel used in our experiments did not favor the detection of infectious H5N1 virus in water (by comparison with water alone). The animals probably filtered and concentrated the RNA to some extent but also probably released some nucleic acids since the initially clean water was slightly contaminated afterwards, and that virus RNA was detected in mussels exposed to infected ones. Our experiments suggest that mussels may be able to at least release nucleic acids in the environment. If they released some infectious virus, it was below the detection threshold of our technique.

Histopathology suggested that the virus was not replicating in mussels and thus that the detection of infectious particles in these molluscs was probably only the result of their natural capacity to filter water. However, this observation may also only be the result of the lower

464 sensitivity of the immunohistochemical method compared to qRT-PCR.Fish (*Betta splendens*)
465 and tadpoles carried detectable HPAI H5N1 virus particles for 1 day only, while infectious
466 particles were isolated from the seeded water until day 2. Interestingly, fish and tadpoles as
467 well as water specimens tested positive by qRT-PCR for 20 days while RNA persisted for a
468 maximum length of 9 days in aquariums containing only rain water (experiment A.1). After 1
469 day of transit of the infected fish in clean water, the RNA was transmitted to exposed fish for
470 only a short period of time. But surprisingly, the initially non-contaminated water tested
471 positive by qRT-PCR for an additional 5 days. The histopathology analyses did not show the
472 presence of virus antigen in the animal tissues tested, suggesting that the virus was not
473 replicating in these tissues. Nevertheless, because of the limited sensitivity of this method,
474 this result should be interpreted with caution. Fish and tadpoles seemed to be able to
475 concentrate the RNA but not the infectious virus in their organs, and to efficiently protect this
476 RNA from decay. These animals also released nucleic acids in water, allowing the detection
477 of H5N1 virus by qRT-PCR for longer periods. Their gills probably acted as filtration systems
478 while the RNA detected in their intestines was probably only the result of the passage of the
479 RNA through the digestive tract, presumably together with food. The detection of nucleic
480 acids but not of infectious virus in fish's organ tissues, including in brain, could be the result
481 of a contamination with nucleic acids from contaminated water during the delicate dissection
482 of the tiny animals, although the animals were all washed in sterile distilled water before the
483 dissection.
484 As often demonstrated through the years, aquatic waterfowl such as ducks, when infected, can
485 shed large amount of virus in their feces, saliva and nasal discharge, all of these potentially
486 resulting in environmental contamination [23,35]. Indeed, in several instances, environmental
487 surfaces, including water, were found to be contaminated by HPAI H5N1 virus during or after
488 outbreaks in poultry [9,10,17,33,35]. Thus, as shown in our study in waters heavily

contaminated by the virus, aquatic molluscs or fish could be passive carriers of avian influenza H5N1 virus and may potentially contaminate domestic or wild birds but also human hosts if correct cleaning and cooking conditions are not applied prior to consumption.

It should be noted that even though this study was meant to reproduce as faithfully as possible the real field conditions, our experiments differed from those by many elements, including the nature of the inoculum. While most environmental materials are contaminated by faeces, saliva, or other organic secretions, our inoculum was amnio-allantoic fluid. In the field, however, it is noteworthy that influenza viruses are protected by organic materials such as nasal secretions or faeces, which may increase their resistance to physical and chemical inactivation [23].

Although in our experimental study HPAI H5N1 infectious virus could not be detected in environmental water and mud from pond and lake origins, we cannot exclude the possibility that the virus could survive in different areas where physico-chemical and microbiological parameters could differ. Indeed, previous studies suggested that even minor fluctuations in temperature, pH and salinity at levels normally encountered in natural aquatic habitats may enhance or diminish environmental persistence [8]. In addition, we may not have selected for our experiments the strains that had the best fitness to persist in the environment. The persistence of viral RNA for periods of 2 weeks in environmental materials is an indicator that at some time, even for a short period, infectious particles were present. Thus, we can not rule out the risk of human contamination from the environment, especially since this risk was suggested and reported in several investigations [4,6,9,22,36-38]. A contaminated environment could provide a continuing source of virus, and restricted access of human and animals to potentially contaminated ponds and lakes should be recommended during and after outbreaks in addition to information regarding the potential risk encountered during collection and consumption of aquatic molluscs or fish. In particular, bathing or swimming activities in

514 contaminated ponds in Cambodia have been clearly identified as a risk factor for human
515 contamination by H5N1 virus [4,22]. Additional factors explaining survival and persistence of
516 HPAI viruses in the environment are still to be elucidated, but our results, along with previous
517 data, support the idea that environmental surveillance is of major relevance for avian
518 influenza control programs.

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524
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526

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650 **Figure legends**

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652 **Figure 1. Design of experiments C and laboratory results to assess the role of bivalves**
653 **(mussels) in the transmission cycle of H5N1 virus in water.**

654 The 3 horizontal rectangles represent different types of water in which mussels were
655 immersed: contaminated vs non-contaminated. Black-filled lines represent mussels immersed
656 in contaminated water from day 0 (D0). White-filled lines represent naïve mussels immersed
657 in non-contaminated water from day 0. The two experiments are discernable and identified as
658 C.1 and C.2 (a, b and c). Water samples were collected daily from day 0 (D0) to day 9 (D9).
659 Numbers not included in boxes correspond to viral loads measured in the water samples.
660 When these numbers are displayed in white color, this indicates that infectious virus was also
661 detected. When the numbers are written in black color, this means that the virus was not
662 recovered after inoculation into eggs.
663 Stars indicate collection of mussels' organs for testing. White stars correspond to detection of
664 infectious virus. Black stars indicate that H5N1 virus was not recovered after inoculation into
665 embryonated eggs. Numbers included in black boxes correspond to average viral loads
666 measured in mussels that were immersed in contaminated water. Numbers in white boxes
667 correspond to average viral loads measured in mussels that were immersed in clean water.
668 When the numbers included in the boxes are displayed in white color, this indicates that
669 infectious virus was also detected. When the numbers are written in black color, this means
670 that the virus was not recovered after inoculation into eggs.

671

672 **Figure 2. Design of experiments D and laboratory results to assess the role of *Betta***
673 ***splendens* fish in the transmission cycle of H5N1 virus in water.**

The 3 horizontal rectangles represent different types of water in which animals were immersed: contaminated vs non-contaminated. Tadpoles (T) were immersed along with the male fighting fish (F) in contaminated water. Black-filled lines represent the male fighting fish and the tadpoles immersed in contaminated water from day 0 (D0). At day 5, male contaminated fish were placed over-night in clean water before being exposed to non-contaminated females in clean water. White-filled lines represent naïve female fighting fish immersed in non-contaminated water from day 0. Stars indicate collection of fish and tadpoles (when present) for testing. The two experiments are discernable and identified as D.1 and D.2. White stars correspond to detection of infectious virus in fish. Black stars indicate that H5N1 virus was not recovered from fish's organs after inoculation into embryonated eggs. Numbers included in black boxes correspond to average viral loads measured in fish (F) and tadpoles (T) that were immersed in contaminated water. Numbers in white boxes correspond to average viral loads measured in female fighting fish that were immersed in clean water and exposed to contaminated male fish.

Water samples were collected from contaminated water (experiment D.1) from day 0 (D0) up to day 20 (D20). Water samples were collected from non-contaminated water (experiment D.2) from day 0 (D0) to day12 (D12). The values presented in italic correspond to the viral load measured in water samples. When the numbers in italic are displayed in white color, this indicates that infectious virus was also detected. When the numbers in italic are written in black color, this means that the virus was not recovered after inoculation into eggs.

Figure 3. **Survival of infectious particles and persistence of virus RNA in simple biotopes (experiments A)**

A.1: only water of various origins maintained at 25°C and inoculated to a final concentration of 5 10^4 EID50/mL with H5N1 virus of animal or avian origin.

699 A.2:water and mud containing an estimated final concentration of virus of avian origin of
700 5 10^4 EID50/mL water maintained at 25°C (A.2.1) or various concentrations of viruses of
701 avian and human origins and maintained at various temperatures (A.2.2).
702
703 A: Avian origin strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain.
704 H: Human origin strain stands for the A/Cambodia/408008/2005 strain.
705 *last day of the corresponding experiment at which samples could be collected and tested.
706
707 **Figure 4: Survival of infectious particles and persistence of virus RNA in complex**
708 **biotopes (experiments B)**
709 B.1: complex biotopes inoculated with virus of avian or human origins at various final
710 concentrations and maintained at 25°C.
711 B.2: complex biotopes inoculated with virus of avian or human origins at various final
712 concentrations and maintained at various temperatures.
713
714 A: Avian origin strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain.
715 H: Human origin strain stands for the A/Cambodia/408008/2005 strain.
716 *last day of the corresponding experiment at which samples could be collected and tested.
717
718 **Figure 5**: **Survival of infectious particles and persistence of virus RNA in water and**
719 **fauna in experiments C and D**
720
721 C: water inoculated with the virus of human origin, at a final estimated concentration of 5 10^4
722 EID50/mL, maintained at 25°C and containing mussels.

723 D: water inoculated with the virus of avian origin, at a final estimated concentration of 2 10^2

724 EID50/mL, maintained at 17°C and containing fighting fish and tadpoles .

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726 *last day of the corresponding experiment at which samples could be collected and tested.

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748 **Tables**

749 **Table 1. Experimental conditions used for the study in simple (A) and complex (B)**

750 **biotopes.**

Series	#		Virus origin[a]	Virus concentration (EID50/mL water)	T°	Water origin	Mud origin	Flora/ fauna
Simple biotopes (A)	**A.1.**		Avian	5 10^4	25	Pond 1 Pond 2 Rain	NA	No
			Human	5 10^4	25	Pond 1 Pond 2 Lake Rain	NA	No
	A.2.	**A.2.1.**	Avian	5 10^4	25	Pond 1 Pond 2 Lake	Pond 1 Pond 2 Lake	No
		A.2.2.	Avian	5 10^2	**22** 25 **32** **34**	Pond 2 Pond 1 Pond 2 Pond 1	Pond 2 Pond 1 Pond 2 Pond 1	No
			Human	5 10^3	25 **32**	Lake	Lake	No
Complex biotopes (B)	**B.1**		Avian	5 10^2	25	Pond 1	Pond 1	Guppies Snails Clams Plants
				5 10^4		Lake	Lake	Guppies Snails Clams Mussels Plants
			Human	5 10^3	25	Lake	Lake	Guppies Tadpoles Plants
				5 10^4				Guppies Clams Plants
	B.2		Avian	5 10^2	**22** **32** **34**	Pond 2 Pond 1	Pond 2 Pond 1	Guppies Snails Clams Plants Guppies Snails Clams Plants
			Human	5 10^3	**32**	Lake	Lake	Guppies Tadpoles Plants

751 [a] Avian origin strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain. Human origin strain stands for the
752 A/Cambodia/408008/2005 strain.
753 [b] T° = Temperature (°C).
754

755

Supplementary material

Supplementary Figure 1. Water temperature measured during experiments D

Supplementary Table 1: Physico-chemical and microbiological parameters measured in water samples prior to experimental contamination.

Supplementary Table 2. Survival of infectious particles and persistence of virus RNA in water specimens of various origins.

Supplementary Table 3. Survival of infectious particles and persistence of virus RNA in mud specimens of various origins.

Supplementary Table 4. Survival of infectious particles and persistence of virus RNA in presence of aquatic flora and fauna.

Supplementary Table 5. Viral load (number of H5 RNA copies/g) measured in different mussel organs obtained in experiment C.2 (virus A/Cambodia/408008/2005).

Supplementary Table 6: Viral load (number of H5 RNA copies/g) in different fish and tadpole organs obtained from experiments D (virus A/chicken/Cambodia/LC1AL/2007)

Supplementary Table 7: Survival of infectious particles and persistence of virus RNA in aquatic environments: compiled data.

Figure 1.

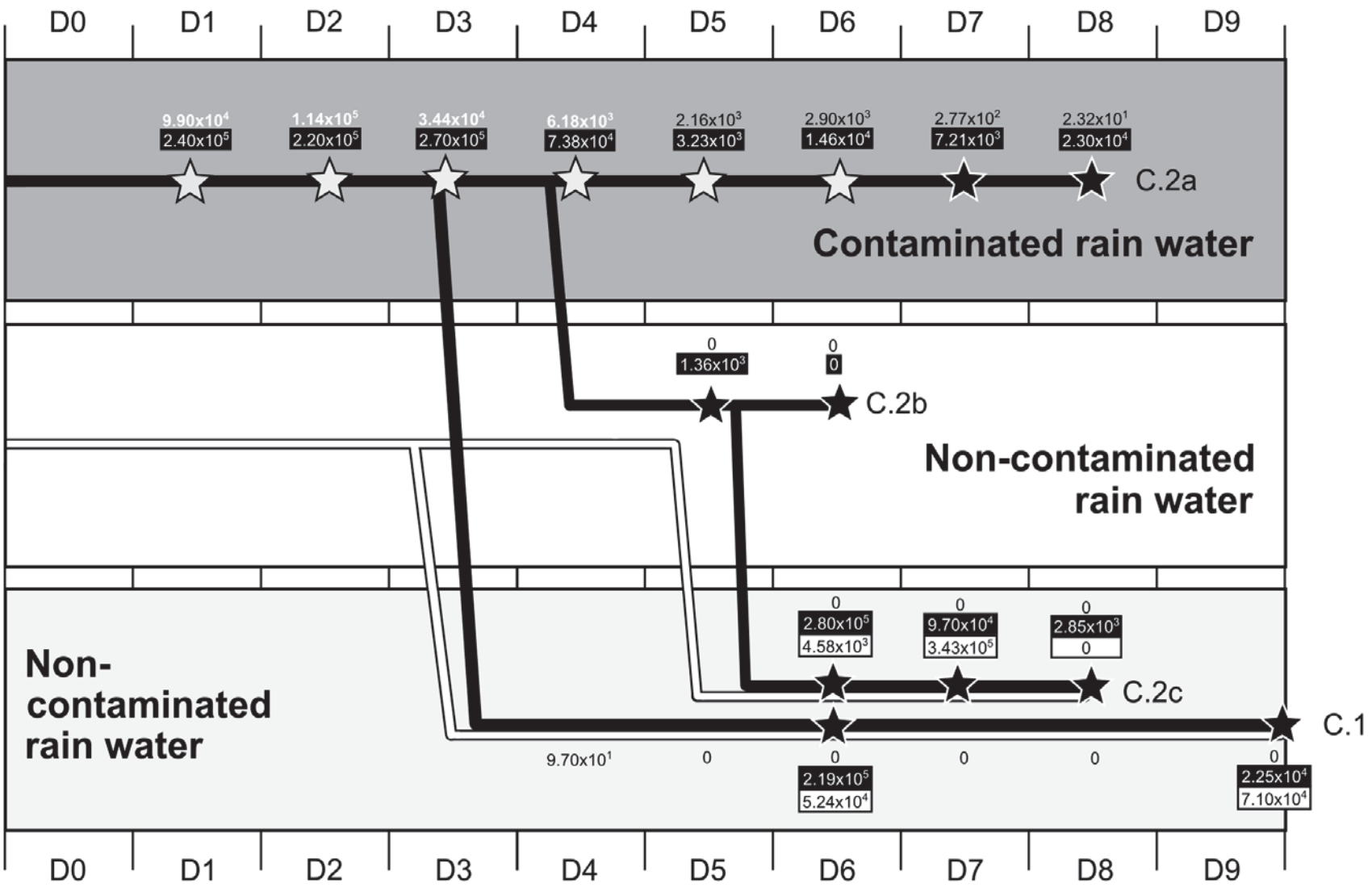

D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
9.90x10^4
2.40x10^5
1.14x10^5
2.20x10^5
3.44x10^4
2.70x10^5
6.18x10^3
7.38x10^4
2.16x10^3
3.23x10^3
2.90x10^3
1.46x10^4
2.77x10^2
7.21x10^3
2.32x10^1
2.30x10^4
C.2a
Contaminated rain water
0
1.36x10^3
0
0
C.2b
Non-contaminated rain water
0
2.80x10^5
4.58x10^3
0
9.70x10^4
3.43x10^5
0
2.85x10^3
0
C.2c
Non-contaminated rain water
9.70x10^1
0
0
2.19x10^5
5.24x10^4
0
0
0
0
2.25x10^4
7.10x10^4
C.1

Figure 2

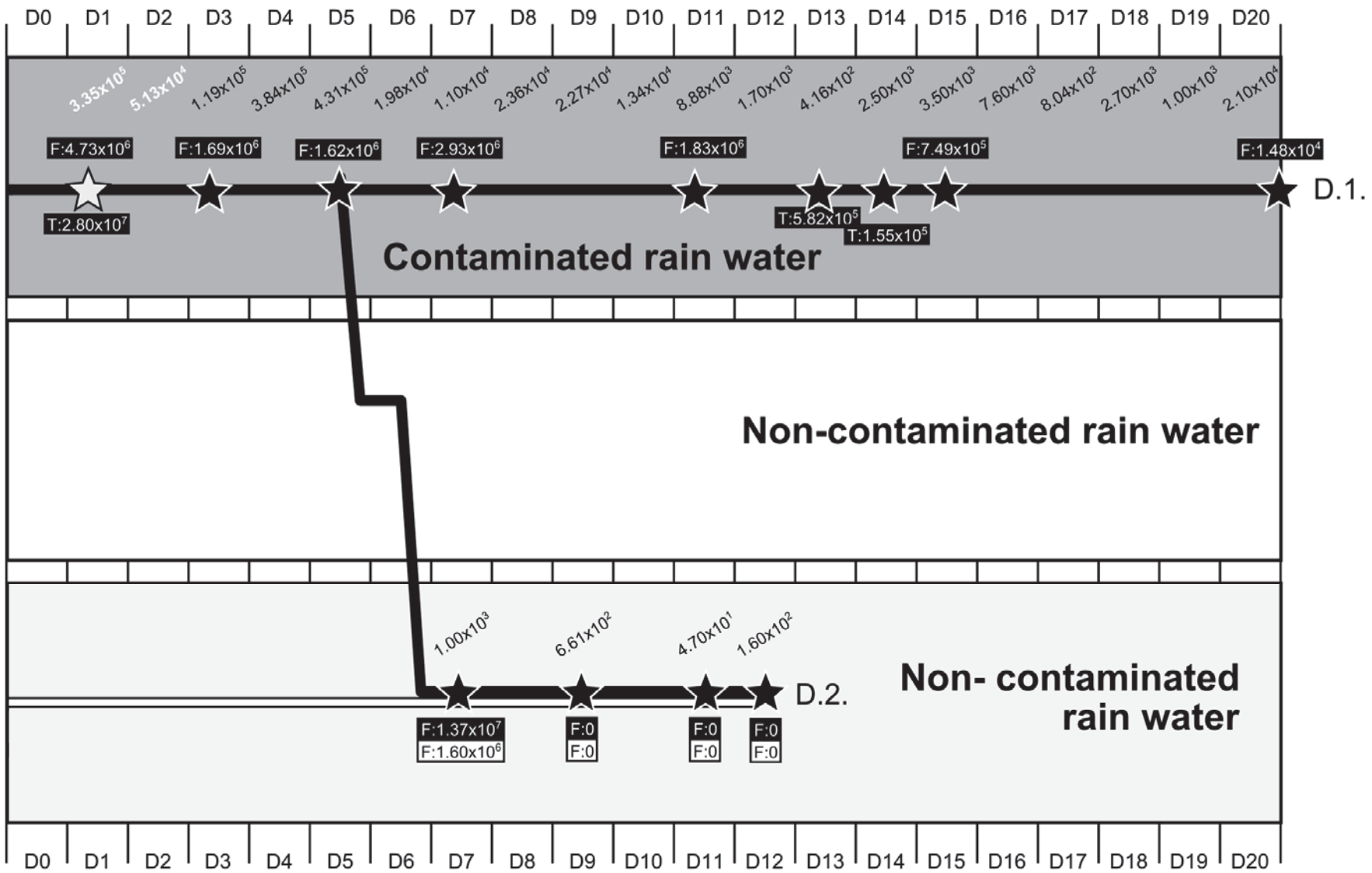

D0
D1
D2
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13
D14
D15
D16
D17
D18
D19
D20
3.35x10^5
5.13x10^4
1.19x10^5
3.84x10^5
4.31x10^5
1.98x10^4
1.10x10^4
2.36x10^4
2.27x10^4
1.34x10^4
8.88x10^3
1.70x10^3
4.16x10^2
2.50x10^3
3.50x10^3
7.60x10^3
8.04x10^2
2.70x10^3
1.00x10^3
2.10x10^4
F:4.73x10^6
F:1.69x10^6
F:1.62x10^6
F:2.93x10^6
F:1.83x10^6
F:7.49x10^5
F:1.48x10^4
D.1.
T:2.80x10^7
T:5.82x10^5
T:1.55x10^5
Contaminated rain water
Non-contaminated rain water
1.00x10^3
6.61x10^2
4.70x10^1
1.60x10^2
D.2.
Non- contaminated rain water
F:1.37x10^7
F:1.60x10^6
F:0
F:0
F:0
F:0
F:0
F:0

Figure 3

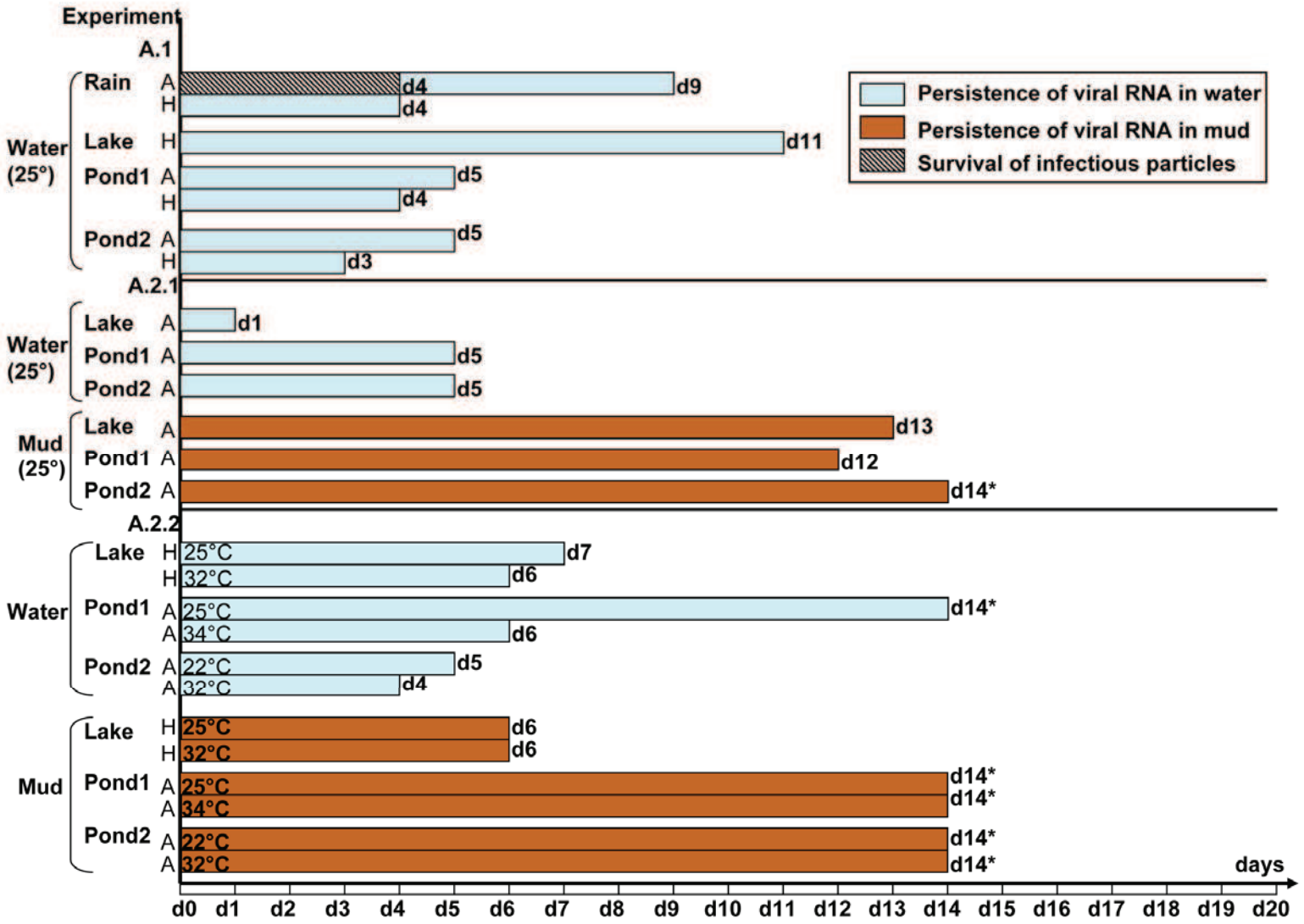

Experiment
A.1
Rain
Water (25°)
Lake
Pond1
Pond2
A.2.1
Water (25°)
Mud (25°)
A.2.2
Water
Mud
Persistence of viral RNA in water
Persistence of viral RNA in mud
Survival of infectious particles
d4
d9
d11
d5
d3
d1
d13
d12
d14*
25°C
32°C
34°C
22°C
d7
d6
days
d0 d1 d2 d3 d4 d5 d6 d7 d8 d9 d10 d11 d12 d13 d14 d15 d16 d17 d18 d19 d20

Figure 4

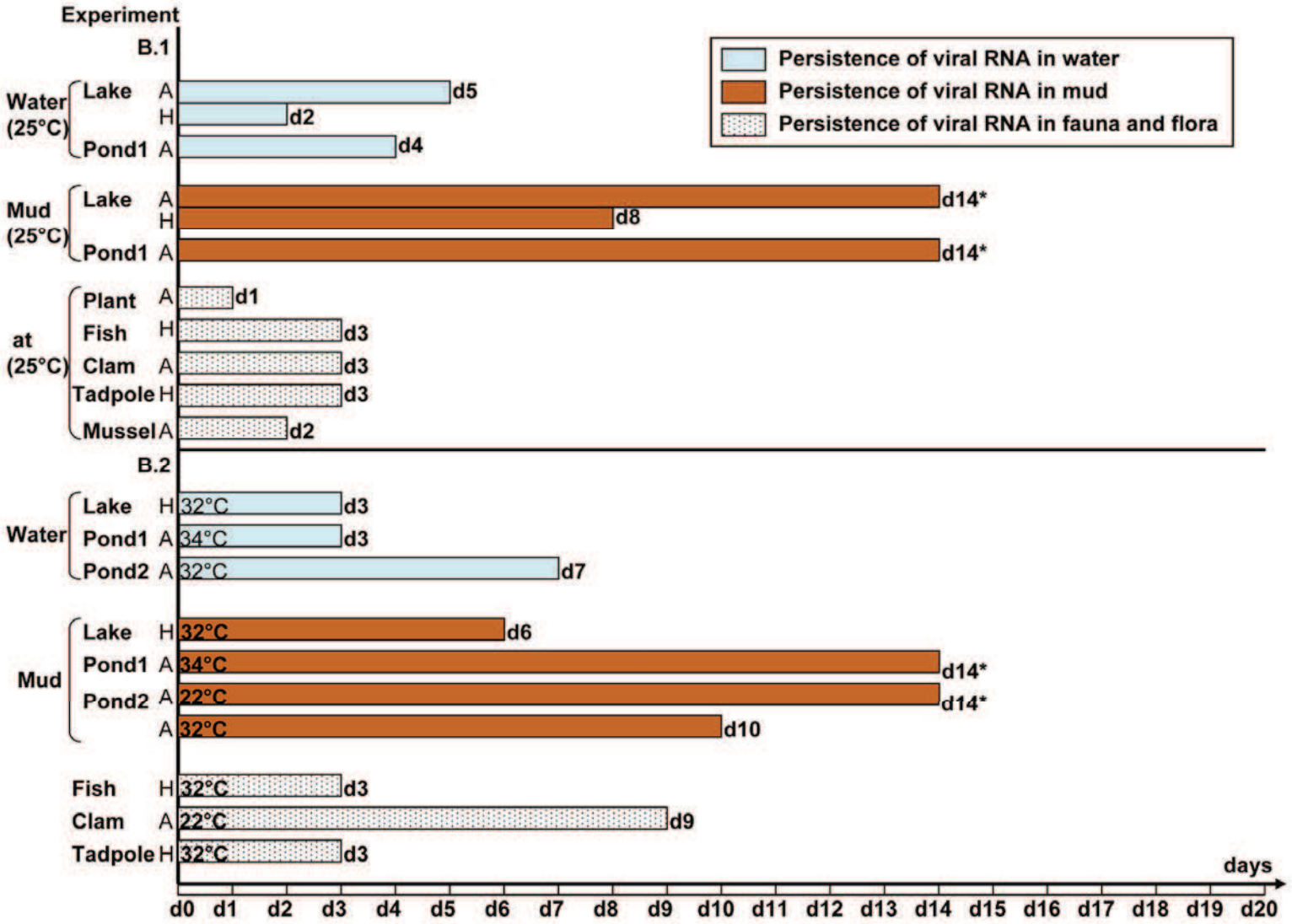
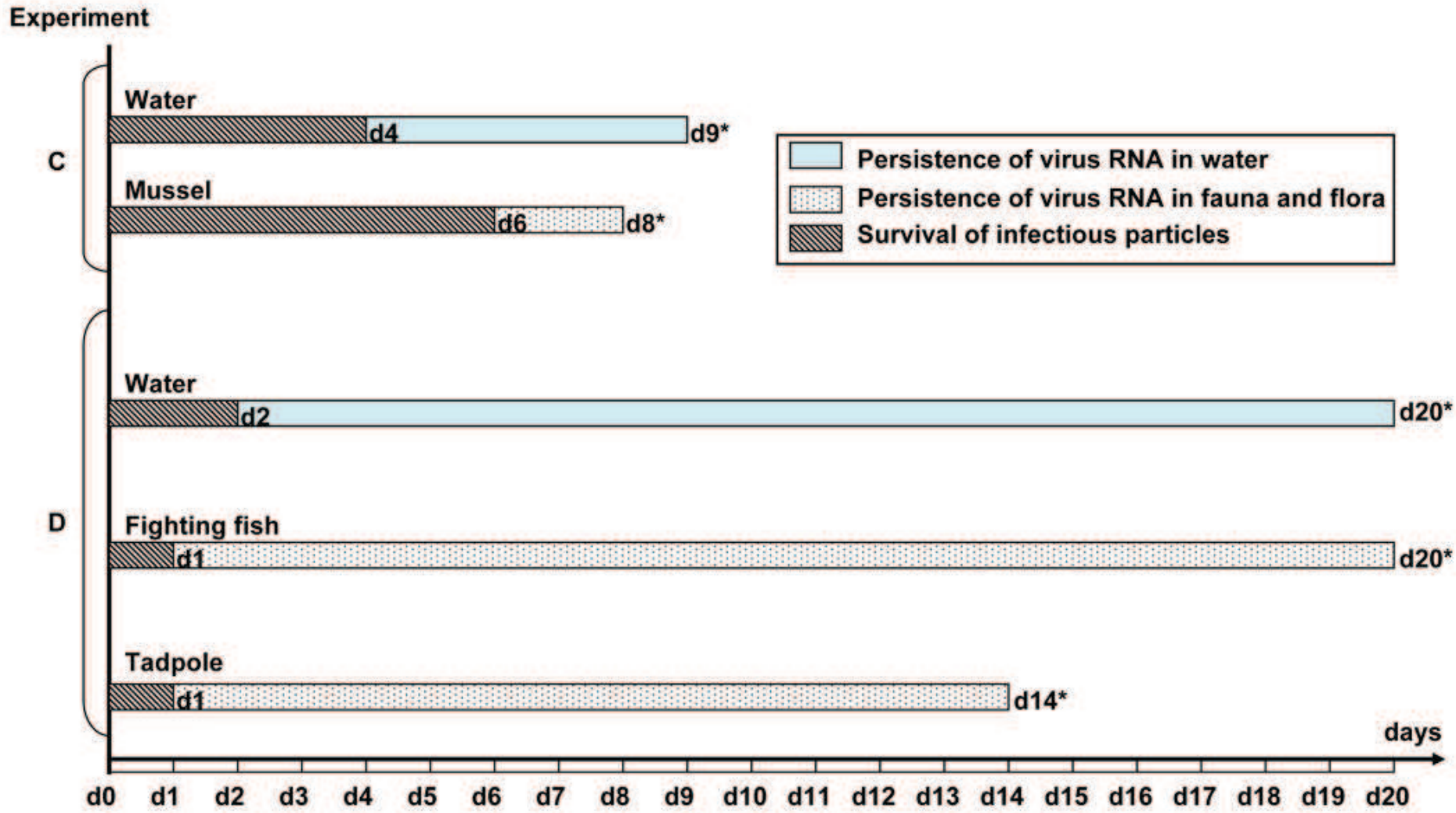
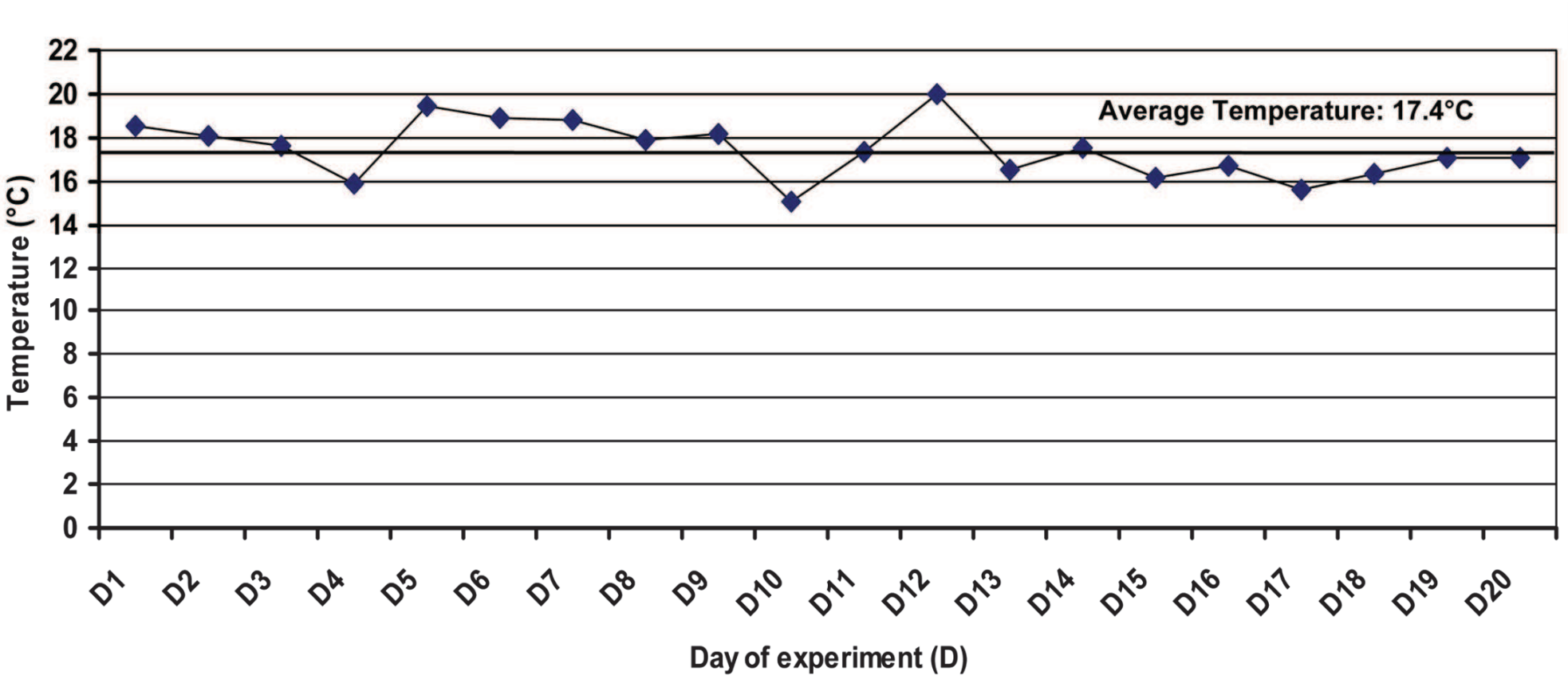

Experiment
B.1
Water (25°C)
Lake A d5
H d2
Pond1 A d4
Mud (25°C)
Lake A d14*
H d8
Pond1 A d14*
at (25°C)
Plant A d1
Fish H d3
Clam A d3
Tadpole H d3
Mussel A d2
B.2
Water
Lake H 32°C d3
Pond1 A 34°C d3
Pond2 A 32°C d7
Mud
Lake H 32°C d6
Pond1 A 34°C d14*
Pond2 A 22°C d14*
A 32°C d10
Fish H 32°C d3
Clam A 22°C d9
Tadpole H 32°C d3
Persistence of viral RNA in water
Persistence of viral RNA in mud
Persistence of viral RNA in fauna and flora
days
d0 d1 d2 d3 d4 d5 d6 d7 d8 d9 d10 d11 d12 d13 d14 d15 d16 d17 d18 d19 d20

Figure 5


Experiment
C
Water
d4
d9*
Mussel
d6
d8*
Persistence of virus RNA in water
Persistence of virus RNA in fauna and flora
Survival of infectious particles
D
Water
d2
d20*
Fighting fish
d1
d20*
Tadpole
d1
d14*
days
d0 d1 d2 d3 d4 d5 d6 d7 d8 d9 d10 d11 d12 d13 d14 d15 d16 d17 d18 d19 d20

Supplementary Figure 1



Supplementary Table 1: Physico-chemical and microbiological parameters measured on water samples prior to experimental contamination.

		Water origin			
		Pond 1	**Pond 2**	**Lake**	**Rain**
Parameters measured on collection sites	**Temperature** (°C)	34	31.7	33.4	18.5
	Conductivity (µS)	60	260	154	/
Physico-chemical analysis in laboratory on samples collected and strored	**Turbidity** (NTU)	112	6	35	6
	pH	7.50	7.45	7.75	8
	Chloride (mg/L)	10	40	63	42
	Ammonium (mg/L)	0.23	0.25	0.04	0.15
	Nitrite (mg/L)	0.06	0.16	0.14	0
	Nitrate (mg/L)	0.34	4.28	1.94	0.5
	Hardness (mg/L)	50	85	95	125
	Total Chlorine (mg/L)	0.08	0.05	0.06	0.05
	Iron (mg/L)	0	0	0.04	0.09
	Total Nitrogen (mg/L)	0	2.2	1.6	1.9
	Sulphate (mg/L)	0	5	6	4
	Sodium (mg/L)	0	4	3	21
	Chemical Oxygen Demand (mg/L)	372	350	13	0
	Phosphate (mg/L)	0	0	2.4	2.2
Bacteriological analysis	**Total aerobic plate count at 37°C-24h** (CFU/mL)	+++ (uncountable)	580	+++ (uncountable)	400
	Total aerobic plate count at 22°C-72h (CFU/mL)	+++ (uncountable)	852	+++ (uncountable)	8600
	Total Coliforms (CFU/100mL)	$2.9\ 10^4$	356	$3\ 10^4$	3000
	Thermotolerant Coliforms (CFU/100mL)	$2.2\ 10^4$	278	$4.4\ 10^3$	2700
	Enterococcus faecalis (CFU/100mL)	0	32	20	260
	Sulphite reducing anaerobies (CFU/20mL)	200	48	40	<1

Supplementary Table 2. Survival of infectious particles and persistence of virus RNA in water specimens of various origins.

Water origin	Series #[a]	Virus origin[b]	Virus concentration (EID50/mL water)	T°	Mud	Flora/fauna	Survival of infectious particles in water (days)	Persistence of viral RNA in water (days)	N# viral RNA copies /mL of water
Rain	**A.1**	Avian	$5\ 10^4$	25	No	No	**4**	**9**	$\mathbf{1.30\ 10^1}$
		Human	$5\ 10^4$	25	No	No	0	**4**	$\mathbf{4.20\ 10^0}$
	C	Human	$5\ 10^4$	25	No	**Yes**	**4**	**9***	$\mathbf{5.50\ 10^2}$
	D	Avian	$\mathbf{2\ 10^2}$	**17**	No	**Yes**	**2**	**20***	$\mathbf{2.10\ 10^4}$
Lake	**A.1**	Human	$5\ 10^4$	25	No	No	0	**11**	$\mathbf{2.22\ 10^2}$
	A.2.1	Avian	$5\ 10^4$	25	**Yes**	No	0	**1**	$\mathbf{1.55\ 10^2}$
	A.2.2	Human	$\mathbf{5\ 10^3}$	25	**Yes**	No	0	**7**	$\mathbf{3.22\ 10^0}$
				32	**Yes**	No	0	**6**	$\mathbf{1.05\ 10^1}$
	B.1	Avian	$5\ 10^4$	25	**Yes**	**Yes**	0	**5**	$\mathbf{1.56\ 10^1}$
		Human	$\mathbf{5\ 10^3}$	25	**Yes**	**Yes**	0	**7**	$\mathbf{9.02\ 10^0}$
			$5\ 10^4$	25	**Yes**	**Yes**	0	**2**	$\mathbf{4.75\ 10^1}$
	B.2	Human	$\mathbf{5\ 10^3}$	**32**	**Yes**	**Yes**	0	**3**	$\mathbf{1.09\ 10^1}$
Pond 1	**A.1**	Avian	$5\ 10^4$	25	No	No	0	**5**	$\mathbf{2.00\ 10^0}$
		Human	$5\ 10^4$	25	No	No	0	**4**	$\mathbf{2.22\ 10^0}$
	A.2.1	Avian	$5\ 10^4$	25	**Yes**	No	0	**5**	$\mathbf{3.50\ 10^0}$
	A.2.2	Avian	$\mathbf{5\ 10^2}$	25	**Yes**	No	0	**14***	$\mathbf{5.20\ 10^1}$
				34	**Yes**	No	0	**6**	$\mathbf{2.02\ 10^2}$
	B.1	Avian	$\mathbf{5\ 10^2}$	25	**Yes**	**Yes**	0	**4**	$\mathbf{1.28\ 10^1}$
	B.2	Avian	$\mathbf{5\ 10^2}$	**34**	**Yes**	**Yes**	0	**3**	$\mathbf{1.11\ 10^1}$
Pond 2	**A.1**	Avian	$5\ 10^4$	25	No	No	0	**5**	$\mathbf{8.00\ 10^0}$
		Human	$5\ 10^4$	25	No	No	0	**3**	$\mathbf{3.96\ 10^1}$
	A.2.1	Avian	$5\ 10^4$	25	**Yes**	No	0	**5**	$\mathbf{9.16\ 10^1}$
	A.2.2	Avian	$\mathbf{5\ 10^2}$	**22**	**Yes**	No	0	**5**	$\mathbf{1.82\ 10^2}$
				32	**Yes**	No	0	**4**	$\mathbf{1.78\ 10^1}$
	B.2	Avian	$\mathbf{5\ 10^2}$	**22**	**Yes**	**Yes**	0	**2**	$\mathbf{1.70\ 10^3}$
				32	**Yes**	**Yes**	0	**7**	$\mathbf{7.88\ 10^2}$

[a] Series numbers as defined in Table 1. A = Simple biotopes, with A.1 = only water, no mud, A.2 = water and mud at 25°C with the standard inoculum dose of $5\ 10^4$ EID50/mL water (A.2.1), and at various temperatures with different inoculum doses (A.2.2). B = Complex biotopes including the presence of flora/fauna, at 25°C (B.1) and other temperatures (B.2).

[b] Avian strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain. Human strain stands for the A/Cambodia/408008/2005 strain.

T° = Temperature (°C).

*last day of the corresponding experiment at which samples could be collected and tested.

Supplementary Table 3. Survival of infectious particles and persistence of virus RNA in mud specimens of various origins.

Mud origin	Series #[a]	Virus origin[b]	Virus concentration (EID50/mL water)	T°	Flora/fauna	Survival of infectious particles in mud (days)	Persistence of viral RNA in mud (days)	N# viral RNA copies /g of mud
Lake	**A.2.1**	Avian	$5\ 10^4$	25	No	0	**13**	**$1.26\ 10^4$**
	A.2.2	Human	**$5\ 10^3$**	25	No	0	**6**	**$1.20\ 10^3$**
				32	No	0	**6**	**$4.30\ 10^3$**
	B.1	Avian	$5\ 10^4$	25	**Yes**	0	**14***	**$1.30\ 10^4$**
		Human	**$5\ 10^3$**	25	**Yes**	0	**8**	**$1.09\ 10^3$**
			$5\ 10^4$	25	**Yes**	0	**1**	**$1.60\ 10^4$**
	B.2	Human	**$5\ 10^3$**	**32**	**Yes**	0	**6**	**$1.13\ 10^3$**
Pond 1	**A.2.1**	Avian	$5\ 10^4$	25	No	0	**12**	**$1.26\ 10^2$**
	A.2.2	Avian	**$5\ 10^2$**	25	No	0	**14***	**$2.23\ 10^2$**
				34	No	0	**14***	**$1.53\ 10^2$**
	B.1	Avian	**$5\ 10^2$**	25	**Yes**	0	**14***	**$2.60\ 10^2$**
	B.2	Avian	**$5\ 10^2$**	**34**	**Yes**	0	**14***	**$4.45\ 10^2$**
Pond 2	**A.2.1**	Avian	$5\ 10^4$	25	No	0	**14***	**$2.77\ 10^3$**
	A.2.2	Avian	**$5\ 10^2$**	**22**	No	0	**14***	**$1.79\ 10^3$**
				32	No	0	**14***	**$5.50\ 10^1$**
	B.2	Avian	**$5\ 10^2$**	**22**	**Yes**	0	**14***	**$3.05\ 10^2$**
				32	**Yes**	0	**10**	**$1.29\ 10^2$**

[a] Series numbers as defined in Table 1. A = Simple biotopes, with A.1 = only water, no mud, A.2 = water and mud at 25°C with the standard inoculum dose of $5\ 10^4$ EID50/mL water (A.2.1), and at various temperatures with different inoculum doses (A.2.2). B = Complex biotopes including the presence of flora/fauna, at 25°C (B.1) and other temperatures (B.2).

[b] Avian strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain. Human strain stands for the A/Cambodia/408008/2005 strain.

T° = Temperature (°C).

*last day of the corresponding experiment at which samples could be collected and tested.

Supplementary Table 4. Survival of infectious particles and persistence of virus RNA in presence of aquatic flora and fauna.

Flora/fauna	Series #[a]	Virus origin[b]	Virus concentration (EID50/mL water)	T°	survival of infectious particles in flora/fauna (days)	Persistence of viral RNA in flora/fauna (days)	N# viral RNA copies/g (organs/plant)
Plants	**B.1**	Avian	$5\ 10^2$	25	0	0	
			$5\ 10^4$	25	0	**1**	**$1.20\ 10^4$**
		Human	$5\ 10^3$	25	0	0	0
			$5\ 10^4$	25	0	0	0
	B.2	Avian	$5\ 10^2$	**22**	0	0	0
				32	0	0	0
				34	0	0	0
		Human	$5\ 10^4$	25	0	0	0
Guppies	**B.1**	Avian	$5\ 10^2$	25	0	0	0
			$5\ 10^4$	25	0	0	0
		Human	$5\ 10^3$	25	0	**3**	**$9.33\ 10^3$**
			$5\ 10^4$	25	0	0	
	B.2	Avian	$5\ 10^2$	**22**	0	0	
				32	0	0	
				34	0	0	
		Human	$5\ 10^3$	**32**	0	**3**	**$5.29\ 10^4$**
Snails	**B.1**	Avian	$5\ 10^2$	25	0	0	
			$5\ 10^4$	25	0	0	
	B.2	Avian	$5\ 10^2$	**22**	0	0	
				32	0	0	
				34	0	0	
Clams	**B.1**	Avian	$5\ 10^2$	25	0	0	
			$5\ 10^4$	25	0	**3***	**$5.70\ 10^4$**
		Human	$5\ 10^4$	25	0	0	
	B.2	Avian	$5\ 10^2$	**22**	0	**9***	**$9.08\ 10^2$**
				32	0	0	
				34	0	0	
Tadpoles	**B.1**	Human	$5\ 10^3$	25	0	**3**	**$8.08\ 10^3$**
	B.2	Human	$5\ 10^3$	**32**	0	**3**	**$1.59\ 10^4$**
	D.1	Avian	$2\ 10^2$	**17**	**1**	**14***	**$1.12\ 10^6$**
Mussels	**B.1**	Avian	$5\ 10^4$	25	0	**2**	**$3.30\ 10^3$**
	C	Human	$5\ 10^4$	25	**6**	**8***	**$2.25\ 10^4$**
Fighting fish	**D**	Avian	$2\ 10^2$	**17**	**1**	**20***	**$1.48\ 10^4$**

[a] Series numbers as defined in Table 1. A = Simple biotopes, with A.1 = only water, no mud, A.2 = water and mud at 25°C with the standard inoculum dose of $5\ 10^4$ EID50/mL water (A.2.1), and at various temperatures with different inoculum doses (A.2.2). B = Complex biotopes including the presence of flora/fauna, at 25°C (B.1) and other temperatures (B.2).
[b] Avian strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain. Human strain stands for the A/Cambodia/408008/2005 strain.
[c] T° = Temperature (°C).
*last day of the corresponding experiment at which samples could be collected and tested.

Supplementary Table 5. Viral load (number of H5 RNA copies/g) measured in different mussel organs obtained in experiment C.2 (virus A/Cambodia/408008/2005).

		Mussel Organs			
	Day of incubation	**Gills**	**Intestine**	**Digestive gland**	**Remaining carcass**
Mussel immersed in contaminated water (M-Inf^{a}) (experiment C.2a)	M-Inf^{a}* Day 1	$1.70\ 10^{6}$	$2.30\ 10^{5}$	$3.20\ 10^{4}$	$2.40\ 10^{5}$
	M-Inf^{a}* Day 2	$2.20\ 10^{5}$	$9.94\ 10^{4}$	$1.73\ 10^{4}$	$4.91\ 10^{3}$
	M-Inf^{a}* Day 3	$2.70\ 10^{5}$	$6.96\ 10^{2}$	$3.27\ 10^{5}$	$1.27\ 10^{5}$
	M-Inf^{a}* Day 4	$7.50\ 10^{2}$	$2.10\ 10^{4}$	$1.14\ 10^{4}$	$7.38\ 10^{4}$
	M-Inf^{a*} Day 5	$3.23\ 10^{3}$	$1.09\ 10^{3}$	$6.97\ 10^{2}$	$3.74\ 10^{2}$
	M-Inf^{a*} Day 6	Negative	Negative	Negative	$1.46\ 10^{4}$
	M-Inf^{a} Day 7	$7.21\ 10^{3}$	$1.85\ 10^{3}$	$9.37\ 10^{2}$	$5.79\ 10^{3}$
	M-Inf^{a} Day 8	$2.30\ 10^{4}$	$2.10\ 10^{3}$	$1.14\ 10^{4}$	$1.15\ 10^{4}$
Mussel from contaminated water transferred to non-contaminated water (M-Inf^{b}) (experiment C.2b)	M-Inf^{b} Day 5	$7.37\ 10^{2}$	$1.04\ 10^{3}$	$1.36\ 10^{3}$	Negative
	M-Inf^{b} Day 6	Negative	Negative	Negative	Negative
Mussel from contaminated water transferred to non-contaminated with exposed mussel (M-Inf^{c}) (experiment C.2c)	M-Inf^{c} Day 6	$4.20\ 10^{3}$	$2.80\ 10^{5}$	Negative	$5.69\ 10^{3}$
	M-Inf^{c} Day 7	$1.39\ 10^{4}$	$8.28\ 10^{3}$	$5.95\ 10^{3}$	$9.70\ 10^{4}$
	M-Inf^{c} Day 8	$6.43\ 10^{2}$	Negative	$5.35\ 10^{2}$	$2.85\ 10^{3}$
Mussel exposed to contaminated mussel (M-exp) (experiment C.2c)	M-Exp Day 6	$1.13\ 10^{3}$	$5.80\ 10^{1}$	$3.47\ 10^{3}$	$4.58\ 10^{3}$
	M-Exp Day 7	$2.04\ 10^{4}$	$2.74\ 10^{4}$	$1.14\ 10^{4}$	$3.43\ 10^{5}$
	M-Exp Day 8	Negative	Negative	Negative	Negative

* Detection of infectious virus particles

Supplementary Table 6. Viral load (number of H5 RNA copies/g) in different fish and tadpole organs obtained from experiments D (virus A/chicken/Cambodia/LC1AL/2007)

		Organs					
	Day of incubation	**Gills**	**Intestine**	**scales**	**Fins**	**Brain**	**Remaining carcass**
Fish immersed in contaminated water (F-Inf[a]) (experiment D1)	F-Inf[a]* Day1	$4.73\ 10^{6}$	$1.85\ 10^{4}$	$4.04\ 10^{4}$	$1.09\ 10^{5}$	$1.85\ 10^{5}$	$3.37\ 10^{3}$
	F-Inf[a] Day3	$1.05\ 10^{6}$	$3.84\ 10^{4}$	$4.34\ 10^{3}$	$1.69\ 10^{6}$	$4.76\ 10^{3}$	$4.24\ 10^{3}$
	F-Inf[a] Day5	$2.72\ 10^{4}$	$1.00\ 10^{5}$	$3.94\ 10^{4}$	$1.62\ 10^{6}$	$1.10\ 10^{6}$	$2.10\ 10^{5}$
	F-Inf[a] Day7	$2.82\ 10^{4}$	$2.93\ 10^{6}$	$1.03\ 10^{4}$	$1.17\ 10^{4}$	$1.08\ 10^{5}$	$5.85\ 10^{5}$
	F-Inf[a] Day11	$1.83\ 10^{6}$	$7.02\ 10^{5}$	$3.69\ 10^{4}$	$1.21\ 10^{5}$	$4.11\ 10^{5}$	$5.09\ 10^{4}$
	F-Inf[a] Day15	$7.49\ 10^{5}$	$1.19\ 10^{5}$	$7.55\ 10^{4}$	$1.00\ 10^{5}$	$6.74\ 10^{4}$	$1.54\ 10^{4}$
	F-Inf[a] Day20	Negative	$5.42\ 10^{3}$	$1.48\ 10^{4}$	Negative	Negative	Negative
Tadpole immersed in contaminated water (T-Inf) (experiment D1)	T-Inf* Day1	$2.80\ 10^{7}$	$6.83\ 10^{6}$	NA	NA	NA	$1.79\ 10^{7}$
	T-Inf Day13	$1.53\ 10^{5}$	$2.72\ 10^{5}$	NA	NA	NA	$5.82\ 10^{5}$
	T-Inf Day14	$1.12\ 10^{5}$	$9.83\ 10^{4}$	NA	NA	NA	$1.55\ 10^{5}$
Fish from contaminated water transferred to non-contaminated water (F-Inf[b]) (experiment D2)	F-Inf[b] Day7	$3.60\ 10^{6}$	$5.50\ 10^{6}$	$2.80\ 10^{5}$	$1.90\ 10^{3}$	$1.37\ 10^{7}$	$2.01\ 10^{3}$
Fish exposed to contaminated fish (F-exp) (experiment D2)	F-Exp Day7	$6.60\ 10^{3}$	$3.40\ 10^{4}$	$1.64\ 10^{4}$	$2.66\ 10^{5}$	$1.60\ 10^{6}$	$1.40\ 10^{5}$

* Detection of infectious virus particles
NA: not available (no organ tested).

Supplementary Table 7: Survival of H5N1 infectious particles and persistence of viral RNA in aquatic environments: compiled data.

Series #[a]	Virus origin[b]	Viral concentration (EID50/mL water)	T°	Water origin	Survival of infectious particles in water (days)	Persistence of viral RNA in water (days)	N# of viral RNA copies /mL of water	Mud origin	Survival of infectious particles in mud (days)	Persistence of viral RNA in mud (days)	N# of RNA copies /g of mud	Flora/fauna	Survival of infectious particles in flora/fauna (days)	Persistence of viral RNA in flora/fauna (days)	N# of RNA copies /g
A.1	Avian	$5\ 10^4$	25	Pond 1	0	5	$2.00\ 10^0$	NA	NA	NA	NA	NA	NA	NA	NA
				Pond 2	0	5	$8.00\ 10^0$	NA	NA	NA	NA	NA	NA	NA	NA
				Rain	4	9	$1.30\ 10^1$	NA	NA	NA	NA	NA	NA	NA	NA
	Human	$5\ 10^4$	25	Pond 1	0	4	$2.22\ 10^0$	NA	NA	NA	NA	NA	NA	NA	NA
				Pond 2	0	3	$3.96\ 10^1$	NA	NA	NA	NA	NA	NA	NA	NA
				Lake	0	11	$2.22\ 10^2$	NA	NA	NA	NA	NA	NA	NA	NA
				Rain	0	4	$4.20\ 10^0$	NA	NA	NA	NA	NA	NA	NA	NA
A.2.1	Avian	$5\ 10^4$	25	Pond 1	0	5	$3.50\ 10^0$	Pond 1	0	12	$1.26\ 10^2$	NA	NA	NA	NA
				Pond 2	0	5	$9.16\ 10^1$	Pond 2	0	14*	$2.77\ 10^3$	NA	NA	NA	NA
				Lake	0	1	$1.55\ 10^2$	Lake	0	13	$1.26\ 10^4$	NA	NA	NA	NA
A.2.2	Avian	$5\ 10^2$	22	Pond 2	0	5	$1.82\ 10^2$	Pond 2	0	14*	$1.79\ 10^3$	NA	NA	NA	NA
			25	Pond 1	0	14*	$5.20\ 10^1$	Pond 1	0	14*	$2.23\ 10^2$	NA	NA	NA	NA
			32	Pond 2	0	4	$1.78\ 10^1$	Pond 2	0	14*	$5.50\ 10^1$	NA	NA	NA	NA
			34	Pond 1	0	6	$2.02\ 10^2$	Pond 1	0	14*	$1.53\ 10^2$	NA	NA	NA	NA
	Human	$5\ 10^3$	25	Lake	0	7	$3.22\ 10^0$	Lake	0	6	$1.20\ 10^3$	NA	NA	NA	NA
			32	Lake	0	6	$1.05\ 10^1$	Lake	0	6	$4.30\ 10^3$	NA	NA	NA	NA
B.1	Avian	$5\ 10^2$	25	Pond 1	0	4	$1.28\ 10^1$	Pond 1	0	14*	$2.60\ 10^2$	Plants	0	0	0
												Guppies	0	0	0
												Snails	0	0	0
												Clams	0	0	0
		$5\ 10^4$	25	Lake	0	5	$1.56\ 10^1$	Lake	0	14*	$1.30\ 10^4$	**Plants**	0	**1**	$1.20\ 10^4$
												Guppies	0	0	0
												Snails	0	0	0
												Clams	0	**3***	$5.70\ 10^4$
												Mussels	0	**2**	$3.30\ 10^3$
	Human	$5\ 10^3$	25	Lake	0	7	$9.02\ 10^0$	Lake	0	8	$1.09\ 10^3$	Plants	0	0	0
												Guppies	0	**3**	$9.33\ 10^3$
												Tadpoles	0	**3**	$8.08\ 10^3$

		5 10^4	25	**Lake**	0	**2**	**4.75 10^1**	**Lake**	0	**1**	**1.60 10^4**	Plants	0	0	0
												Guppies	0	0	0
												Clams	0	0	0
B.2	Avian	**5 10^2**	**22**	**Pond 2**	0	**2**	**1.70 10^3**	**Pond 2**	0	**14***	**3.05 10^2**	Plants	0	0	0
												Guppies	0	0	0
												Snails	0	0	0
												Clams	0	**9***	**9.08 10^2**
			32	**Pond 2**	0	**7**	**7.88 10^2**	**Pond 2**	0	**10**	**1.29 10^2**	Plants	0	0	0
												Guppies	0	0	0
												Snails	0	0	0
												Clams	0	0	0
			34	**Pond 1**	0	**3**	**1.11 10^1**	**Pond 1**	0	**14***	**4.45 10^2**	Plants	0	0	0
												Guppies	0	0	0
												Snails	0	0	0
												Clams	0	0	0
	Human	**5 10^3**	**32**	**Lake**	0	**3**	**1.09 10^1**	**Lake**	0	**6**	**1.13 10^3**	Plants	0	0	0
												Guppies	0	**3**	**5.29 10^4**
												Tadpoles	0	**3**	**1.59 10^4**
C	Human	5 10^4	25	**Rain**	**4**	**9***	**5.50 10^2**	NA	NA	NA	NA	**Mussels**	**6**	**8***	**2.25 10^4**
D	Avian	**2 10^2**	**18-22**	**Rain**	**2**	**20***	**2.10 10^4**	NA	NA	NA	NA	**Tadpoles**	**1**	**14***	**1.12 10^6**
												Fighting fish	**1**	**20***	**1.48 10^4**

[a] Series numbers as defined in Table 1. A = Simple biotopes, with A.1 = only water, no mud, A.2 = water and mud at 25°C with the standard inoculum dose of 5 10^4 EID50/mL water (A.2.1), and at various temperatures with different inoculum doses (A.2.2). B = Complex biotopes including the presence of flora/fauna, at 25°C (B.1) and other temperatures (B.2).
[b] Avian strain stands for the A/Chicken/Cambodia/LC1AL/2007 strain. Human strain stands for the A/Cambodia/408008/2005 strain.
T° = Temperature (°C).
*last day of the corresponding experiment at which samples could be collected and tested.

4. Conclusions de l'étude

Cette étude montre que la persistance de virus H5N1 infectieux ne dépasse pas 4 jours, et seulement dans l'eau de pluie. Aucun virus vivant n'a pu être détecté dans l'eau de mare ou de lac ou dans les boues même lorsque des concentrations élevées de virus ont été utilisées. Toutefois, l'ARN viral persiste jusqu'à 20 jours dans l'eau de pluie et 7 jours dans de l'eau de mare et de lac. Dans plusieurs cas, l'ARN viral a aussi été détecté dans des échantillons de boue, jusqu'à 14 jours post-contamination. Tous ces résultats confirment les données de l'étude précédente qui montrait que l'ARN viral H5N1 était retrouvé dans des échantillons environnementaux collectés dans les alentours des zones d'épidémie de virus H5N1 au Cambodge.

De plus, dans le cadre de cette étude, des virus infectieux et de l'ARN viral ont été détectés dans quelques cas dans la faune et la flore aquatiques, en particulier dans les mollusques bivalves (moules) et des poissons (poissons combattants), bien que ces organismes semblent être essentiellement des transporteurs passifs du virus plutôt que des hôtes permettant au virus de se répliquer.

La persistance de l'ARN viral sur une période de 2 semaines dans l'environnement est préoccupante. Nous ne pouvons pas exclure le risque de contamination humaine à partir de l'environnement, surtout quand ce risque a déjà été suggéré et rapporté par plusieurs enquêtes précédentes. L'environnement contaminé pourrait fournir une source continue de transmission du virus, et la restriction de l'accès aux étangs, mares ou lacs potentiellement contaminés doit être recommandé dans les zones où le virus circule. Des restrictions doivent également être appliquées à la consommation de mollusques aquatiques et de poissons.

Bien que plusieurs facteurs de survie et de persistance des virus H5N1 HP dans l'environnement restent encore à élucider, et particulièrement difficile à contrôler dans des conditions de laboratoire, nos résultats, avec les données précédentes, soutiennent l'idée que la surveillance de l'environnement est très importante pour les programmes de contrôle et de lutte contre la grippe aviaire.

CONCLUSION et PERSPECTIVES

Les principaux résultats obtenus dans ce travail de thèse contribuent à mieux comprendre la persistance du virus influenza aviaire H5N1 HP dans l'environnement, et particulièrement dans l'environnement aquatique, d'un pays tropical comme le Cambodge.

Dans ce travail de thèse, la technique de concentration du virus influenza H5N1 dans l'eau été mise au point, validé et appliqué dans la détection du virus H5N1 HP dans de grands volumes d'eau de surface (Chapitre 1). La technique repose sur un principe d'adsorption-élution sur laine de verre suivie d'une concentration secondaire par précipitation à l'aide de polyéthylène glycol. Le seuil de détection déterminé dans cette étude permet de mettre en évidence la présence, dans dix litres d'eau, d'une quantité de virus avoisinant la dose infectante pour un canard. Les rendements déterminés avec différents types d'eau (de pluie et de lac) combinés à la valeur du seuil de détection permettront d'interpréter les futurs résultats avec une plus grande précision. De plus, les rendements ne varient pas selon la saison durant laquelle le prélèvement a été réalisé ni selon le site géographique de prélèvement de l'échantillon, ce qui permet une large utilisation de la technique. Il est important de noter que les différentes étapes de concentration n'ont pas endommagé les virus, ces derniers ayant conservé leur pouvoir infectieux. La technique de concentration testée ici a montré son efficacité et sa fiabilité, elle pourra donc être utilisée dans les investigations épidémiologiques des foyers d'IAHP à venir, au Cambodge et ailleurs. Des analyses d'eau prélevée sur des foyers d'anciennes épidémies H5N1 au Cambodge n'ont pas permis de mettre en évidence la présence de virus. Par conséquent, des recherches plus poussées devront donc être menées sur les prochains foyers afin d'apporter de nouvelles données sur l'écologie du virus H5N1 et l'épidémiologie de l'IAHP.

Une technique de concentration, d'identification et de quantification du virus influenza dans la boue a été également développée et validée (Chapitre 2). En parallèle, cinq méthodes d'extraction directe de l'ARN du virus H5N1 à partir de la boue ont été testées avec la

détermination des seuils de détection et le calcul des rendements pour chaque méthode. Le résultat nous montre que quand l'extraction d'acide nucléique se fait directement sur des échantillons de boue, la méthode utilisant QIAamp Viral RNA Mini Kit et Trizol LS donne les meilleurs résultats. Toutefois, quand la technique d'élution/concentration a été réalisée avant l'extraction de l'acide nucléique, le kit MagNA pure LC augmente le performant. Une procédure utilisant des étapes d'élution avec une solution d'extrait de bœuf 10% (pH7) et de concentration avec polyéthylène glycol a été conçue pour détecter les VIA par RT-PCR dans des échantillons de boues.

Face à l'absence de connaissances scientifiques sur de nombreux aspects de l'écologie des virus IAHP, et en particulier sur le virus H5N1, les méthodes de concentration, d'identification et de quantification du virus influenza H5N1 dans l'eau et dans la boue peuvent à présent être utilisées pour mieux comprendre la perpétuation d'année en année du virus et sa survie possible dans l'environnement.

Ces méthodes ont été utilisées dans les analyses des prélèvements d'eau, de boue de l'environnement naturel collectés lors des investigations épidémiologiques des foyers d'épidémie du virus H5N1 AIHP au Cambodge (Chapitre 3) et dans des expériences réalisées sur des biotopes artificiels dans le cadre de notre étude (Chapitre 4).

Au cours de 4 épidémies de virus H5N1 HP chez des volailles au Cambodge entre 2007 et 2010, de nombreux échantillons environnementaux des zones d'épidémie ont montré une contamination par le virus. La présence de l'ARN viral a été détectée dans des échantillons de poussière, de boue, de sol et d'eau.

De nouvelles recherches devraient donc être menées sur les prochains foyers épizootiques afin d'apporter de nouvelles données sur l'écologie du virus H5N1 et l'épidémiologie de l'IAHP.

Dans ce travail de thèse, l'observation de la persistance du virus H5N1 HP dans des systèmes expérimentaux d'environnements aquatiques artificiels (Chapitre 4) nous montre que les souches Cambodgiennes de virus H5N1 d'origine humaine et aviaire ne pouvaient persister

que pendant de très courtes durées dans l'eau de pluie bien que l'ARN viral soit présent entre 7 et 20 jours selon le type d'eau et les température testées. Dans la boue de mare et de lac, les particules virales infectieuses ne pourraient pas survivre très longtemps bien que l'ARN puisse persister au moins 2 semaines. En outre, la présence de moules et / ou d'autres types de faune ainsi que la flore aquatique n'ont aucune influence sur la persistance du virus dans tous les divers types d'eau testés. Mais on retrouve chez ces animaux aquatiques de l'ARN viral jusqu`au 20ème jour. Du virus infectieux a été également isolé dans les premiers jours suivant l'inoculation virale. Ce résultats incitent à la prudence quant à la consommation d'animaux aquatiques, en particulier des mollusques quand ces derniers sont collectés dans des lacs ou des mares situées dans des zones où le virus H5N1 HP a été déclaré chez les volailles domestiques.

En conclusion, au cours des épidémies à virus H5N1, l'environnement entourant ces zones est contaminé par le virus et peut agir comme une source potentielle de contamination humaine et / ou animale. La limitation de l'accès à l'eau potentiellement contaminée doit être recommandée autour des foyers épidémiques. La surveillance de l'environnement est nécessaire pour un programme de lutte contre la grippe aviaire qui doit en outre prendre en considération les mesures de désinfection de l'environnement.

Il est nécessaire d'approfondir les connaissances sur les voies potentielles de transmission du virus H5N1 aux humains à partir des environnements contaminés sans omettre d'étudier les pratiques potentiellement à risque de contamination dans les populations humaines. Des études de contamination environnementale approfondies, y compris la contamination dans les marchés vendant des volailles vivantes, couplées à des études comportementales et sero-épidémiologiques chez les individus exposés pourront contribuer à améliorer cette compréhension.

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Etude de la survie du virus H5N1 dans des environnements aquatiques artificiels reproduisant les biotopes naturels du Cambodge, pays d'endémie en zone tropicale

Résumé

Bien que la persistance du virus H5N1 dans l'environement soit possible, il n'existe aucune méthode bien définie, standardisée, pour détecter du virus à partir de l'eau, du sol ou de la boue. De plus, il n'y a que très peu de données relatives au rôle du virus H5N1 dans l'environnement en pays tropical. Dans ce travail, des méthodes de concentration, d'identification, et de quantification du virus influenza dans l'eau et dans la boue ont été développées, validées puis utilisées pour l'analyse de prélèvements de l'environnement collectés au cours d'investigations d'épidémies de virus H5N1 au Cambodge et pour l'étude de la survie du virus influenza aviaire dans des biotopes aquatiques artificiels reproduisant le plus possible les conditions naturelles observées dans les mares ou les lacs au Cambodge.
L'ARN du virus H5N1 été détecté dans 19% des échantillons environnementaux de terrain collectés au décours des épidémies. Des particules virales infectieuses ont été isolées dans un échantillon d'eau d'une ferme. Dans des systèmes expérimentaux, le virus H5N1 infectieux persiste seulement 4 jours dans l'eau de pluie. Mais l'ARN viral peut encore être détecté jusqu'à 20 jours dans l'eau de pluie et 7 jours dans l'eau de mare ou de lac. Dans la boue, les particules virales infectieuses ne semblent pas pouvoir survivre bien que l'ARN puisse persister au moins 2 semaines. La faune et la flore aquatique n'ont aucune influence sur la persistance du virus infectieux dans l'eau. Ces organismes semblent être essentiellement des concentrateurs et des transporteurs passifs du virus plutôt que des hôtes autorisant la réplication du virus. Nos résultats montrent que l'environnement au cours d'épidémies est fortement contaminé par le virus H5N1 et pourrait constituer une source potentielle de contamination humaine et/ou animale. Une restriction de l'accès à l'eau potentiellement contaminée doit être recommandée autour des foyers épidémiques. La surveillance de l'environnement doit être intégrée dans les programmes de lutte contre la grippe aviaire qui doivent par conséquent prendre en considération des mesures de désinfection de l'environnement.

Mots clés : virus H5N1, environnement, eau, boue, biotopes aquatiques, Cambodge.

Influenza A(H5N1) survival in artificial aquatic biotopes reproducing the natural environments of Cambodia, endemic country in the tropic

Summary

Although the persistence of the H5N1 virus in the environment is possible, there is no well-defined and standardized method for the detection of viruses from water, soil or mud. In addition, there is very little data available regarding the role of H5N1 virus in the environment in the tropics. In this work methods of concentration, identification, and quantification of influenza viruses in water, mud and soils have been developed, validated and used to test environmental samples collected following H5N1 outbreaks in Cambodia and to analyze samples obtained during experiments in artificial aquatic biotopes aiming to reproduce as faithfully as possible the characteristics observed in ponds and lakes in Cambodia. The H5N1 viral RNA was detected in 19% of environmental samples. Among these, infectious viral particles were isolated in a single water sample. In experimental systems, the infectious H5N1 virus survived only 4 days in the rain water. But viral RNA persisted up to 20 days in rain water and 7 days in pond and lake water. In mud, infectious viral particles did not survive even viral RNA could persist for at least 2 weeks. Aquatic flora and fauna have no influence on infectious H5N1 virus persistence in water. These organisms seem to concentrate and to passively carry the virus but do not allow virus replication. Our results showed that following outbreaks, the environment is widely contaminated by H5N1 virus and therefore can act as a potential source of human and/or animal contamination. Restricted access to potentially contaminated water should be recommended during outbreak episodes. Monitoring the environment is recommended in the effort to fight against avian influenza and measures including environment disinfection should also be considered.

Key words: H5N1 virus, environment, water, mud, aquatic biotopes, Cambodia

ការសិក្សាអំពីការរស់របស់មេរោគH5N1នៅក្នុងមជ្ឈដ្ឋានទឹកសប្បនិមិត្តដែលបង្កើតឡើងក្នុងលក្ខខ័ណ្ឌដូចនៅនឹងបរិស្ថានទឹកធម្មជាតិពិត នៅប្រទេសកម្ពុជាដែលជាប្រទេសនៅក្នុងតំបន់ត្រូពិច មានផ្ទុះជំងឺគ្រុនផ្តាសាយបក្សីប្រភេទ H5N1 ជាប្រចាំ

សង្ខេប

ទោះបីជាយើងបានដឹងថា មេរោគគ្រុនផ្តាសាយបក្សីប្រភេទH5N1 អាចស្ថិតនៅក្នុង បរិស្ថានធម្មជាតិបាន ប៉ុន្តែយើងនៅមិនទាន់មានបច្ចេកទេស ដែលមានលក្ខណៈស្តង់ដា សំរាប់ធ្វើការវិភាគរកមេរោគ នៅក្នុងទឹក នៅក្នុងភក់ឬដី នៅឡើយទេ។ ជាងនេះទៅទៀត ទិន្នន័យស្តីអំពី មេរោគគ្រុនផ្តាសាយបក្សីប្រភេទH5N1 នៅក្នុងបរិស្ថានធម្មជាតិ នៃប្រទេសនៅក្នុងតំបន់ត្រូពិច មានតិចតួចណាស់។

នៅក្នុងនិក្ខេបទនេះ វិធី កំហាប់ ទឹក និង កំហាប់មេរោគ ដើម្បី រកមេរោគ និង បរិមាណនៃមេរោគគ្រុនផ្តាសាយ នៅក្នុងទឹក នៅក្នុងភក់ ត្រូវបានបង្កើតឡើង, ហើយធ្វើការវាយតំលៃប្រសិទ្ធិភាពនៃតេស្ត និងប្រើប្រាស់បច្ចេកទេសនេះ ដើម្បីរកមេរោគH5N1 នៅក្នុង ទឹក ភក់ ដែលយក ពីបរិស្ថានធម្មជាតិ នៅកន្លែងដែលកំពុងមានផ្ទុះ ជំងឺគ្រុនផ្តាសាយបក្សីប្រភេទH5N1នៃប្រទេសកម្ពុជា។ បច្ចេកទេសនេះក៏ត្រូវយកមកប្រើ ដើម្បីរកមេរោគH5N1 នៅក្នុង ទឹក ភក់ ដែលប្រមូលបាន ពីការពិសោធន៍ អំពីការរស់រានរបស់មេរោគH5N1 នៅក្នុង អាងទឹកសប្បនិមិត្ត (biotopes aquatiques) ដែលមានលក្ខណៈស្រដៀងទៅនឹងលក្ខខ័ណ្ឌធម្មជាតិពិតនៃ ស្រះ ឬ បឹង នៅប្រទេសកម្ពុជា ។

យើងរកឃើញមាន ARN មេរោគH5N1 នៅក្នុង 19%នៃវត្ថុវិភាគ ដែលយកចេញ ពីបរិស្ថានធម្មជាតិ នៅកន្លែងដែលកំពុងមានផ្ទុះ ជំងឺគ្រុនផ្តាសាយបក្សី។ ហើយយើងក៏រកឃើញ មេរោគH5N1 នៅរស់ និងដែលអាចបង្កជំងឺចំនួន ១ នៅក្នុងទឹកតួកផងដែរ។ នៅក្នុង ការពិសោធន៍របស់យើង, យើងឃើញថា មេរោគH5N1 នៅរស់បានតែ ៤ថ្ងៃ នៅក្នុង ទឹកភ្លៀង ប៉ុន្តែARN នៃមេរោគ អាចនៅរកឃើញ ក្នុងទឹករហូតដល់ ២០ថ្ងៃ នៅក្នុង ទឹកភ្លៀង និង ៧ថ្ងៃ នៅក្នុង ទឹកស្រះ ឬ បឹង ។ រីឯនៅក្នុងភក់វិញ, យើងមិនឃើញមាន មេរោគH5N1 ដែលអាចនៅរស់ទេ ទោះបីជា ARN នៃមេរោគនេះអាចនៅរកឃើញ ក្នុងភក់ រហូតដល់ ២សប្តាហ៍ក៏ដោយ ។ ចំណែកឯ សត្វនិងរុក្ខជាតិ ក្នុងទឹក គឺមិនមានឥទ្ធិពលទៅលើលទ្ធភាពស្ថិតនៅរបស់មេរោគនៅក្នុងទឹកនោះទេ។ ពពួកសត្វនៅក្នុងទឹក ដូចជាគ្រំ និងត្រី វាគ្រាន់តែអាចផ្ទុក ឬពាំនាំនៅមេរោគH5N1 បណ្តោះអាសន្ន ក្នុងរយៈពេលខ្លី ប៉ុន្តែ វាមិនអាចអោយ មានការរីកលូតលាស់សាយភាយ នៃមេរោគនៅក្នុងសរីរាង្គរបស់វា នោះទេ ។

លទ្ធផលនៃការសិក្សានេះបង្ហាញអោយឃើញថា នៅកន្លែងដែលកំពុងមានផ្ទុះជំងឺគ្រុនផ្តាសាយបក្សីប្រភេទ H5N1, បរិស្ថានធម្មជាតិនៅទីនោះមានមេរោគH5N1 ហើយវាអាចជាប្រភពចំលង មេរោគនេះទៅ មនុស្ស និង សត្វ ។ ដូចនេះយើងត្រូវមានការប្រុងប្រយ័ត្ន ក្នុងតំបន់ដែលមាន ផ្ទុះជំងឺគ្រុនផ្តាសាយបក្សី, យើងគួរជៀសវាង ឬកាត់បន្ថយការចូលទៅលេងទឹក ឬប្រើប្រាស់ ទឹកដែលអាច មានមេរោគ ។ កម្មវិធី ទប់ស្កាត់ការរីករាលដាលនៃជំងឺគ្រុនផ្តាសាយបក្សីត្រូវបន្ថែម ការតាមដាននិងធ្វើការអង្កេត នៅក្នុងបរិស្ថាន ក្នុងតំបន់ដែលមាន ផ្ទុះជំងឺនេះ និង ត្រូវយកចិត្តទុកដាក់ក្នុងការសំអាត និង កំចាត់មេរោគ នៅក្នុងបរិស្ថានផងដែរ ។

ពាក្យគន្លឹះ : មេរោគH5N1, បរិស្ថាន, ទឹក, ភក់, អាងទឹកសប្បនិមិត្ត (biotopes aquatiques), ប្រទេសកម្ពុជា