Potential Role of Fresh Water Apple Snails on H5N1 Influenza Virus Persistence and Concentration in Nature

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[2]

The receptors of AIVconsist of sialic acid (SA) derived from a monosaccharide linked to the galactose of a glycoprotein or a glycolipid embedded in the host cell's membrane and involved in the

eggshells in the aquarium. Mortality was high in the early days but stabilized quickly (Table 1). After three months of rearing, the snails had reached adult size of 1 to 3 cm.

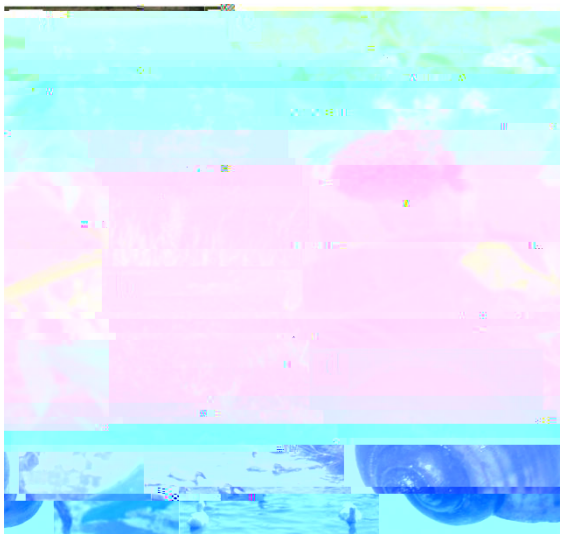

Figure 1: Apple snail and duck natural environment

(a) Free grazing wild ducks in rice fields after harvesting (Thailand).

(b) Free grazing semi-domestic ducks in rice fields after harvesting (Thailand).

(c) Apple snail eggs laid on water grass in natura (Thailand).

(d) Pomaceacanaliculata (Apple snail)

Three plastic containers (39x27x18 cm) were introduced into a poultry isolator in ABSL-3 laboratory and prepared one week before the experiment in order to establish abiotic conditions. Each container contained ten liters of mineral water, stones and gravel, with diffusors to oxygenate the water. Non-hermetic lids closed the containers. The hand-raised snails were introduced into two containers (60 specimens per container), one container remaining only with water, without snails. The temperature was set at 30°C, close to natural conditions, and daily checked (Figure 2).

The same dose of H5N1 virions was added to the three containers and, samples taken from water and snails to test for presence of active virus. Snails were dissected to separately collect different organs including, gills, pseudo-lung, intestines and foot.

Samples (water and snails) were cultured by inoculation into the allantoic fluid of embryonated chicken eggs (fertilized and incubated for 9 to 11 days). Allantoic fluid was then collected and tested by Hemaglutination Test (HT) for the presence of the active virus.

To increase the high sensitivity of the test for the presence of active virus (P>0.95), each water sample was inoculated to four embryonated chicken eggs, as well for snail sample using two embryonated eggs for each sample, leading to an overall sensitivity of the five snails collected with P>0.95. When the outcome was negative, we conducted a new test (blind passage) by inoculating the negative allantoic fluid into a new egg [27]. The result was considered positive when positive HT was observed for at least one of the eggs.

Minimum detectable virus concentration (HT sensitivity) was set up with a P>0.95 of HT after culturing the virus on eggs. For a test using four eggs, the sensitivity u of an egg must be at least 0.52 ($P' = \left[1 - (1 - u)^4\right]$). We evaluated the minimum detectable concentration with a sensitivity>=0.5, by inoculating eggs to assess the percentage of positives. To obtain an accuracy of 0.15 in this assessment, with a type I error of 0.05, it is necessary to inoculate at least 40 eggs 3.

We tested the concentrations 10, 5 and 1 TCID50/ml. The 0.2 ml dose injected into each egg was respectively equal to 2, 1 and 0.2 TCID50. The results are summarized in Table 2[3].

TCID50/ml	Positive[a]	First passage[b]	Dead after 1st day[c]	Dead after 2nd day[d]
10	26/40 (65)	21/40 (52)	1 / 1	19/20d
5	22/40 (55)	17/40 (42)	2 / 2	19/20
1	20/40 (50)	11/40 (27)	1 / 1	7/8

Table 2: Sensitivity Assessment between Hemaglutination test and RT-PCR test.

(a) positive / total tested (%); (b) Number of positive test the first egg inoculation (over 40 done); (c) Number of dead embryo one day after infection / total tested; (d) Number of positive dead embryo two days after infection / total tested

At a starting dose of 2 TCID50 (10 TCID50/ml concentration) and 1 TCID50 (5 TCID50/ml concentration), the virus induced a very high mortality of egg embryos. With a starting dose of 0.2 TCID50 (concentration 1 TCID50/ml), 50% of the results was positive: in the condition of our study, the HT using eggs with two passes was 5 times more accurate than a cell culture test. Test applied to water samples (four eggs with two passes) allowed detecting the virus at a concentration of 10 TCID50/ml with a probability of 0.985, at a concentration of 5 TCID50/ml with a probability of 0.959, and at a concentration of 1 TCID50/ml with a probability of 0.937. The detection test used for snails (two eggs with two passes on five samples) allowed detecting the virus at a concentration 10 TCID50/ml with a probability of 0.995, at a concentration of 5 TCID50/ml with a probability of 0.982, and at a concentration of a 1 TCID50/ml with a probability of 0.969. Virus detection and titration by qRT-PCR were performed on all samples of water and snails, subjected to qRT-PCRto verify the presence of H5N1 viral genetic material and to determine its concentration (H5 Gene [GenBank: EF593102]). The technique required only a minimum of three RNA copies of the target gene [28]. False positives by external contamination may occur. HT gives the best specificity and provides the largest number of true negatives. qRT-PCRallows calculating the concentration of viral particles, active or inactivated. We used the kit SuperScript III OneStep RT-PCR System (Invitrogen®) according to manufacturer's recommendations. The amplicons obtained were analyzed by the program Rotor-Gene Real-Time Analysis Software 6.1®. In the absence of known variability for virus persistence in water, the significance of the difference in virus persistence between the two containers (with and without snails) could be analyzed frR Manalyzed frR Manalyzed frR Manalyzed frR ManalyzeӟDR M verifə prď R Ma a cPd mor_ ofT dμ ℵm ή Ma

(a) H=Hemaglutination; (b) copies / ml; (c) PCR=qRT-PCR; (d) +=number of positive embryonated egg (with four tests for each water sample +- one blind passage for each first negative test); (d) -

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