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Original Article

Avian influenza A H5N1 infections in Bali province, Indonesia: a behavioral, virological and seroepidemiological study

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Background Bali Province was affected by avian influenza H5N1 outbreaks in birds in October 2003. Despite ongoing circulation of the virus, no human infection had been identified by December 2005.

Objectives To assess behavioral patterns associated with poultry rearing in Bali, and to identify potential risk factors for H5N1 infection in humans and in household chickens, ducks and pigs.

Methods A behavioral, virological and seroepidemiologic survey in 38 villages and three live bird markets was completed in December 2005. A multi-stage cluster design was used to select 291 households with 841 participants from all nine districts in Bali. Specimens were collected from participants as well as a maximum of three pigs, chickens and ducks from each household. Eighty-seven market vendors participated, where specimens were collected from participants as well as chickens and ducks.

Results Twenty out of the 38 villages sampled had H5N1 outbreaks. Despite exposure to H5N1 outbreaks, none of the participants from villages or markets were seropositive for H5N1. None of the pigs tested were positive for H5N1. Virus isolation rate in ducks and chicken in markets was higher than in households. Transport of poultry in or out of villages was a risk factor for outbreaks in household chickens and ducks.

Conclusions The study highlighted that the market chain and associated behaviors may play a role in maintaining the virus in household flocks. The study adds evidence that transmission of H5N1 to humans remains a rare event despite high level handling of both healthy and sick birds.

Keywords Avian influenza, H5N1 virus, Indonesia, pigs, risk factors, seroepidemiological study.

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Introduction

Avian influenza H5N1 causing human disease was first reported in Hong Kong in 1997. Widespread outbreaks in poultry were reported in Southeast Asia in late 2003 and 2004 in a number of countries including Thailand, Viet Nam, Cambodia and Indonesia, with zoonotic transmission to humans.[1] In Indonesia, the epidemic of avian influenza H5N1 in birds started in Java Island in August 2003, from where the disease spread to other islands. By the end of 2005, the disease in birds was considered endemic in parts of Java, Sumatra, Kalimantan, Sulawesi and Bali. Human cases of avian influenza H5N1 were first identified in Indonesia in July 2005 and since then an average of three cases continues to be detected per month. Due to international concern resulting from the human avian influenza H5N1 infection in Indonesia, we report on a study conducted in Bali in December 2005 addressing potential risk factors for infection of humans and transmission in animals.

Bali is an island province consisting of nine districts and is located between Java and Lombok Islands. The population of Bali is about 2·85 million people, where the majority of people are farmers. Most households keep a small number of poultry or may even have larger pens for commercial activity. Based on a survey in 2004 conducted by the Provincial Livestock Office, the domestic chicken, duck and other farmed bird population in Bali was estimated at 12 million. Pigs are reared in backyard settings in Bali

province, unlike most other regions in Indonesia, where there is close intermingling of humans, chickens, ducks and pigs. Due to the close interaction between the different species and the widespread outbreaks of avian influenza H5N1 virus, understanding the epidemiology of the disease in this island province was deemed a priority.

Outbreaks of avian influenza H5N1 were first reported in Karangasem district in Bali in October 2003. After that, the disease spread to all nine districts. The disease affected domestic birds including layers, broilers, village chickens, ducks, muscovy, goose, quails and pigeons.[2] In response to the outbreaks, an H5N1 vaccination programme was commenced in June 2004. The government provided 8·6 million doses of vaccine in 2004 and 7·2 million doses in 2005 to cover the chicken and duck population. The vaccine provided was a locally-produced inactivated oil-emulsion vaccine using an Indonesian strain (A/Ck/Legok/2003). The vaccine was manufactured by three companies in Indonesia and was available commercially. The manufacturers and Bali Provincial Livestock Office reported that only large-scale holders purchased the vaccine privately, whereas backyard poultry holders generally depended on government supplies or did not vaccinate at all.

Despite widespread outbreaks of H5N1 in the bird population and the close interaction between people and their farmed birds and pigs in Bali, no human suspect or confirmed H5N1 cases had been identified through the hospital-based surveillance programme at the time this study was carried out at the end of 2005. This study assesses the demographics and behavioral patterns associated with chicken/duck and pig rearing in Bali, as well as risk factors associated with outbreaks of H5N1 in household chickens/ducks. The study attempts to identify the seroprevalence of H5N1 neutralizing antibodies in humans and associated risk factors for human infection. Lastly, the study attempts to identify the prevalence of H5N1 infection in pigs.

Methods

Household survey

We conducted a behavioral, virological and seroepidemiologic survey in December 2005, approximately 18 months after the first chicken outbreaks of H5N1 were identified in Bali Province but 2 years before the first human cases were confirmed in the province. The survey was a household-based cluster survey with the goal of 570 participants: 20 persons in each of the 30 clusters. The sample size was calculated to have a 95% chance of detecting ≥1 seropositive persons assuming 3% seroprevalence of H5N1 antibodies. To identify the villages for inclusion, we used a multi-stage cluster sampling method.[3] Due to resource constraints, in the first stage, we decided to sample intensively in three out of the nine districts in Bali. The criteria for choosing the three districts were that farming practices in the districts were representative of farming practices in Bali and that the three districts were considered highly affected by outbreaks of highly pathogenic avian influenza H5N1 virus based on previous surveillance data.[2] Based on this, Bangli, Karangasem and Tabanan districts (39·9%, 8·5% and 7·7% of villages were respectively affected between October 2003 and September 2004) were selected.

In the second stage, we defined a cluster as a village. The probability of selecting a village (cluster) was proportional to the chicken population size. This method was used as the greater the number of chicken in the population, the greater the risk of infection to human and pigs. The Provincial Livestock Office estimated the size of the chicken population in each village in 2004. In addition to the 30 clusters chosen from the three districts that were to be sampled intensively, we chose the two villages from each of the remaining six districts with the highest chicken populations. This was done to enable us to draw more general conclusions about avian influenza H5N1 prevalence in Bali Province. This brought the total number of clusters to 42.

In the third stage, we used WHO/Expanded Program on Immunization's cluster sampling proximity method.[3] We randomly selected the first household within the cluster and subsequent households were selected by proximity until 20 eligible participants were enrolled in each cluster. From each household, we limited the number of participants to four so that we could sample a minimum of five households in each cluster. For households with more than four eligible persons to be enrolled into the study, interviewers were asked to list all of the eligible persons and then select four by using random number tables.[4]

The survey consisted of interviews with household members with a standardized questionnaire on demographic information, behavioral patterns and contact with animals. A 5-ml blood specimen was collected from human participants. From each household surveyed, we collected serum, throat and nasal swabs from up to three pigs, serum, tracheal and cloacal swabs from a maximum of three chickens and serum, tracheal and cloacal swabs from a maximum of three ducks. To assess risk factors for outbreaks in animals, a village was considered affected by H5N1 outbreaks if it had one or more of the following; (A) PCR-confirmed H5N1 outbreaks in the 4 months prior to the study based on surveillance findings from the Balinese Provincial Livestock Office, (B) H5N1 virus was isolated from chicken/duck samples collected during the study or (C) seropositive chickens/ducks with no history of vaccination AND where one or more households in that village reported sudden mortality of at least 50% of their flocks in the past 18 months.

Market survey

In addition to the household survey, we also surveyed one live bird market from each of the three intensely sampled districts. The main live bird market in each of the three districts was selected for the study, where sampling was conducted once during the same month of the household-based survey. All markets operated daily, had live birds and carcasses for sale, and slaughtering was conducted in the markets. All stalls with chickens/ducks were approached to participate, where questionnaires were administered to stall operators and 5-ml blood specimen collected from the stall operators. A total of 87 market stall operators participated, where 18 participants were from the Karangasem market, 28 were from the Bangli market and 40 were from the Tabanan market. Cloacal and tracheal swabs as well as blood specimens were collected from a maximum of three chickens and three ducks per stall.

Laboratory methods

Human and animal blood specimens were collected in vaccutainers, kept cold and taken to the Provincial Health Laboratory and Disease Investigation Center (DIC) respectively, where they were centrifuged on arrival, sera aliquoted and frozen at −80°C. Chicken and ducks sera were tested by haemagglutination inhibition tests using A/Chicken/Bangli Bali/BBPV6/04 using standard methods[5] and an antibody titer of 1/20 or higher was taken to be a positive result. Human and pig sera were shipped on dry ice to the WHO H5 Reference Laboratory at the University of Hong Kong for detection of neutralizing antibodies to the same virus (above) by microneutralization assay as previously described.[6] Serologic evidence of H5N1 virus infection was defined as an H5N1 neutralizing antibody titer ≥80 for both human and pig sera.

All animals swabs were placed into viral transport medium in sterile tubes in the field, kept cold and transported daily to the Denpasar DIC of the Ministry of Agriculture. All animal swabs and sera were aliquoted and tested at two independent laboratories (DIC and the University of Hong Kong). Chicken and duck swabs were inoculated into 9- to 10-day-old embryonated eggs and those positive in the hemagglutination (HA) assay were tested by Hemagglutination Inhibition test with reference antisera.[6] Pig swabs were inoculated into both embryonated eggs (as for avian swabs) and also into Madin Darby Canine Kidney (MDCK) cells for virus isolation. MDCK cells were examined daily for cytopathic effect and tested by immunofluorescence for influenza A antigen (DAKO, Denmark) when cytopathic effect appears or otherwise at day 7 post-infection.[6] Influenza A virus isolates were genetically sequenced using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Viral RNA was extracted from allantoic fluid of the positive samples using QIAamp Viral RNA mini kit (Qiagen, Chatsworth, CA, USA). One-step RT-PCR was performed using Superscript TM III one-step RT-PCR with Platinum Taq (Invitrogen, USA). All PCR products were purified by QIAquick PCR purification kit (Qiagen). Sequencing was performed by BigDye Terminator V3.1 cycle sequencing kit on ABI PRISM 3700 DNA analyzer (Applied Biosystems, USA). All sequence segments were assembled and aligned by BioEdit, version 7 (USA). Phylogenetic tree was generated by neighbor-joining bootstrap analysis (1000 replicates) using the Kimura two-parameter model in MEGA, version 3.1 (USA).[7]

Statistical analysis, ethics approval and funding source

Individual, household and market questionnaire data were entered into a Microsoft Access database twice by two independent data entry teams. The data were cleaned by reviewing duplicate records, typing errors and by conducting logic checks. Microsoft Excel, EpiInfo (CD, Atlanta, GA, USA) and Stata version 8.0 (Stata Corp. LP, College Station, TX, USA) were used for descriptive and statistical analyses. To assess risk factors associated with likely H5N1 outbreaks in chickens/ducks in households, we estimated odds ratios using bivariate and multivariate logistic regression in stata. We accounted for the cluster effect of households using Stata's cluster option for logistic regression. For the models in the multivariate analysis, variables with a $P \leq 0{\cdot}1$ from the bivariate analyses were included. Written informed consent was obtained from every participant. As advised by the Bali Provincial Health Office, the study was approved by Bali Province Research Ethics Committee, where the Committee stipulated that human participants in the study had to be ≥17 years of age. The field work was funded by the World Health Organization Indonesia Country Office, laboratory testing in Hong Kong funded from Grant AoE/M-12/06 from the University Grants Committee of Hong Kong and the data analysis at the Denpasar Disease investigation centre was funded from their routine service funding.

Results

Household demographic and behavioral survey

The seroepidemiological study had 841 participants from 291 households. The majority of respondents (52%) were between 26 and 45 years of age (mean = 42 years of age), where the age distribution closely reflected the population age distribution. The proportion of male (n = 423) to female (n = 418) participants was similar, also reflecting the underlying population distribution. The majority (73%) of participants completed primary school education, 21% had no education and only 5% had university education.

From the 841 household participants, 26% of participants currently or recently (within last 2 months) worked in poultry-related 'high exposure' occupations (live bird market worker, poultry collector or poultry farmer). From the remaining 74% of participants, the majority were employed as non-poultry agriculture farmers (31%), unemployed (18%) or had other occupations.

In the survey, 263 (90%) households owned chickens/ducks, where the majority owned chickens ($n = 260$). The median number of chicken in households surveyed was 12 (range: 1–30 000). For households with ducks ($n = 68$), the median number of ducks owned was four (range: 1–2000). Even though the majority of households had a small number of chickens/ducks, a few households in the study had commercial production-level chicken and duck populations. 198 households (68%) owned pigs, where the median number of pigs was four (range: 1–102). The majority of households surveyed either had a combination of chickens and pigs (131 households), only chickens (64 households) or a combination of chickens, ducks and pigs (52 households).

Households had multiple uses for their chickens/ducks, including keeping them as pets (92%), for food (87%), to sell (56%) or to sell eggs (35%). The majority of household participants handled, fed and slaughtered chickens/ducks in the 2 months prior to the survey (Table 1). Sixty households (23%) experienced sudden high mortality (≥50% of total population) in their chicken/duck flock in the last 18 months. These households reported disposing of the sick/dead animals by burying them (68%) or burning the carcasses (12%), but very few reported other actions such as eating them, throwing them away or throwing them in the river (5%).

In households with pigs, the majority of households farmed pigs commercially (97%) as well as keeping them for their own consumption (87%). Fifty-four percent of participants reported handling pigs in the last 2 months, 48% cleaned up pig feces and 13% reported slaughtering pigs.

Household chickens and ducks

None of the chickens or ducks sampled in the household survey were positive for H5N1 virus (Table 2). One duck was positive for H4 influenza virus.

From the 263 households with birds, 28% reported vaccinating their birds for H5N1. The majority ($n = 60$, 81%) of households vaccinating for H5N1 reported obtaining the vaccine from government sources. The majority of the vaccinating households (85%) reported vaccinating their flocks between one and three times in the last 18 months; only 15% of households vaccinated more than three times in that period. Increasing numbers of vaccine doses reportedly used in chickens was significantly associated with increasing proportions of seropositive chickens in those flocks ($P < 0{\cdot}001$) (Figure 1). However, even after the administration of three vaccine doses, only 60% of chickens in these flocks were seropositive. Paradoxically, for chickens that received more than three doses of vaccine, a smaller proportion of the flock was seropositive. It is important to note that all of the chickens that received more than three

Table 1. Comparison of behavioral exposures in people surveyed in households and markets

Exposure	Villages, *N* = 841 (%)	Markets, *N* = 87 (%)	*P*-value
Handle live birds	648 (77·1)	76 (87·4)	0·038
Feed birds	611 (72·7)	75 (86·2)	0·008
Clean bird cages	448 (53·3)	55 (63·2)	0·096
Slaughter chickens	463 (55·1)	51 (58·6)	0·6
Slaughter ducks	34 (4)	25 (28·7)	<0·001
Handle bird organs	473 (56·2)	53 (60·9)	0·468
Prepare birds for restaurants	16 (1·9)	4 (4·6)	0·109
Transport birds	180 (21·4)	62 (71·3)	<0·001
Handle bird feces/fertilizer	376 (44·7)	46 (52·9)	0·179
Collect eggs from cages	224 (26·6)	18 (20·7)	0·283
Handle sick/dead birds	119 (14·1)	36 (41·4)	<0·001
Chickens	117 (13·9)	19 (21·8)	0·067
Ducks	8 (1)	19 (21·8)	<0·001
Handle live pigs	454 (54)	48 (55·2)	0·921
Slaughter pigs	106 (12·6)	20 (23)	0·011
Clean up pig faeces	401 (47·7)	32 (36·8)	0·067
Handle sick/dead pigs	35 (4·2)	5 (5·7)	0·677

Table 2. H5N1 virus isolation and H5N1 seroprevalence of humans and animals sampled

Species	Sample type	Village samples positive	Market samples positive	Total samples positive
Human	Serum*	0/841	0/87	0/928
Chicken	Serum†	84/544	7/36	91/580
	Cloacal swab‡	0/521	0/61	0/582
	Tracheal swab‡	0/518	1/63	1/581
Duck	Serum†	25/78	0/32	25/110
	Cloacal swab‡	0/81	1/34	1/115
	Tracheal swab‡	0/82	7/36	7/118
Pig	Serum*	0/344	–	0/344
	Throat/nasal swab‡	0/304	–	0/304
	Throat swab‡	0/35	–	0/35
	Nasal swab‡	0/35	–	0/35

*H5N1 antibody detection by microneutralization test.
†H5N1 antibody detection by haemagglutination inhibition test.
‡H5N1 virus isolation detected by PCR.

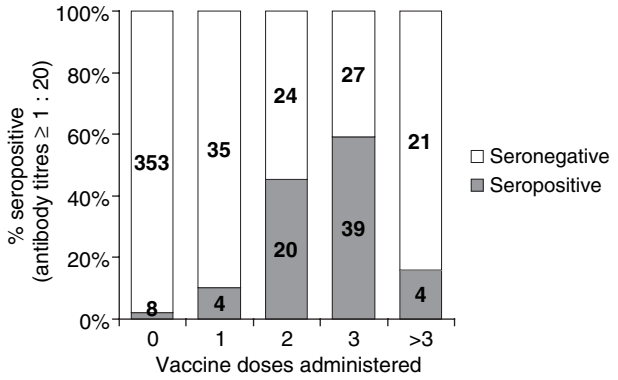

Figure 1. Proportion of chickens seropositive for H5N1 by the haemagglutination inhibition test in households, stratified by number of H5N1 vaccine doses administered in previous 18 months.

doses of vaccine were from one cluster (village flock) in the survey.

Seven out of 22 vaccinated ducks had H5N1 antibody titers ≥20 (32%). Twelve out of 57 non-vaccinated ducks were also seropositive (26%). This contrasts with only eight out of 366 non-vaccinated chickens being seropositive (2%, $P < 0{\cdot}001$).

Villages with H5N1 outbreaks

Twenty out of the 38 villages sampled in the survey had H5N1 outbreaks in chickens/ducks according to the survey definition. Provincial Livestock Office data showed that seven of the villages surveyed had H5N1 outbreaks in the last 4 months (unpublished data). All of these outbreaks were confirmed by RT-PCR and, in four cases, virus isolation. Thirteen other villages were confirmed based on survey definition (C), where chickens/ducks were found to be seropositive without vaccination history and one or more households in the same village had sudden high mortality in their chicken/duck flocks. In these villages, five villages had seropositive chickens without vaccination history, six villages had seropositive ducks without vaccination history and two villages had both seropositive chickens and ducks without vaccination history. Households in all 13 villages reported death in their chicken/duck flocks, where a median of two households (range: 1–4 households) reported sudden mortality in at least 50% of their flocks. None of the villages were confirmed to have recent H5N1 outbreaks using survey definition (B) as all of the household chickens and ducks tested in the survey were found to be negative for H5N1 virus isolation.

Risk factors for H5N1 outbreaks in village and household chicken/ducks

We assessed risk factors for H5N1 outbreaks in household chickens/ducks at village level, where we compared exposures in villages with H5N1 outbreaks ($n = 20$) to villages without outbreaks ($n = 18$). We found that if one or more household in a village transported live chickens/ducks in and out of the village, this increased the probability of H5N1 outbreaks (Table 3). Two variables with $P \leq 0{\cdot}1$ were considered in the multivariate analysis; transportation of birds in/out of villages and sale of eggs by at least one household in village. Transportation of birds in and out of villages remained a significant risk factor after controlling for the other exposure in the multivariate analysis (OR = 2·31, $P = 0{\cdot}046$, CI: 1·02–5·23). We compared households reporting sudden mortality in ≥50% chicken/-duck flocks with households not reporting sudden chicken/duck mortality in the 20 H5N1 outbreak villages (data not shown). Similar to the analyses done at village-level, we found that transportation of live chickens/ducks in and out of the village increased the probability of an outbreak in that specific household (OR = 2·612, $P = 0{\cdot}013$, CI: 1·22–5·57).

Table 3. Comparison of exposures in villages with H5N1 outbreaks ($n = 20$) and in villages with no history of H5N1 outbreaks ($n = 18$)

Exposure	Villages with H5N1 outbreak, *N* = 20 (%)	Villages with no history of H5N1 outbreaks, *N* = 18 (%)	OR*	*P*-value	95% CI*
Household flock size ≥20 chicken/ducks in at least 1 household in village	12 (60)	5 (27·8)	1·85	0·103	0·88–3·89
History of H5N1 bird vaccination in at least 1 household in village	15 (75)	12 (66·7)	1·81	0·217	0·70–4·66
Meat prepared for restaurants in at least 1 household in village	6 (30)	7 (38·9)	0·4	0·39	0·05–3·33
Birds transported in/out of village by at least 1 household in village	18 (90)	9 (50)	2·61	0·013	1·22–5·57
At least 1 household in village keeps birds for sale	20 (100)	16 (88·9)	1·61	0·249	0·72–3·63
At least 1 household in village keeps birds to sell eggs	16 (80)	13 (72·2)	1·91	0·087	0·91–4·03

*OR, odds ratio; CI, confidence interval.

Pigs

Of the 344 pigs sampled in the household survey, 222 pigs (65%) were from 127 households in H5N1 outbreak-confirmed villages and 122 were from 73 households in unconfirmed/non-outbreak villages. None of the pigs surveyed in the households were positive for H5N1 virus (Table 2). Similarly, all were found to be seronegative for H5N1 antibody by microneutralization assay.

Household participants

From the 841 participants in the household survey, 480 were from the 20 villages with H5N1 outbreaks and 361 were from the 18 villages without evidence of H5N1 outbreaks. Fourteen percent ($n = 119$) of the total number of participants reported handling sick/dead chickens or ducks in the last 18 months (Table 1). Of these 61% ($n = 72$) were from villages with H5N1 outbreaks and 39% ($n = 47$) were from villages without evidence of H5N1 outbreaks.

Even though 57% of participants resided in villages with a history of H5N1 outbreaks ($n = 480$) and 14% of all study participants reported handling sick/dead chickens and ducks ($n = 119$), none of the 841 household participants had neutralizing antibodies suggestive of H5N1 virus infection on microneutralization assay (Table 2).

Market survey

From the 99 chicken and duck swabs collected at the markets, one chicken and eight ducks were positive for H5N1 virus (Table 2). All of the virus-positive animals were from two of the three markets surveyed. The rate of virus isolation from markets was significantly higher than that in the villages ($P < 0{\cdot}001$). These H5N1 viruses were confirmed to be highly pathogenic avian influenza H5N1 viruses by sequencing the haemagglutinin cleavage site (QRERRRKKR/G). Results of the phylogenetic analysis of the HA gene of the viruses indicates that the viruses fall within the Indonesian sublineage (clade 2·1). The viruses in each market were genetically heterogenous suggesting that there were multiple virus introductions into each market (Figure 2). One duck in a market was positive for H4 influenza virus.

Eighty-seven stall operators participated in the market survey. The majority reported handling live birds (87%), feeding birds (86%) and transporting birds (71%) (Table 1). Forty-one percent ($n = 36$) of market participants reported handling sick/dead chickens and ducks. These behaviors were more prevalent amongst market vendors compared to community participants ($P < 0{\cdot}05$) (Table 1). Despite the intense handling of healthy and sick chickens and ducks, as well as reporting of symptoms, none of the market participants had neutralizing antibodies suggestive of H5N1 virus infection on microneutralization assay.

Discussion

The ecologic study revealed that the majority of households had backyard poultry and pigs and that mixed farming of chickens, ducks and pigs was commonplace. This is similar to reports elsewhere in Asia.[8] The primary finding of our study was that despite high level handling of birds, including sick/dead birds, and exposure to likely outbreaks of H5N1, none of the participants from either households or markets were seropositive for H5N1. This finding is comparable to a study in Cambodia where despite high level exposure to H5N1, no human seropositivity was observed.[9,10]

Although the household survey suggested evidence of H5N1 outbreaks in the past, none of the sampled chickens or ducks were positive for H5N1 virus isolation. In contrast, two of the three poultry markets had H5N1 virus isolated, where eight of the nine positive birds were ducks. Based on known practices in the market chain in Bali, it is suspected that the ducks sold in live bird markets originate from Java rather than from backyard ducks in Bali. The phylogenetic tree suggests multiple introductions of virus into each market. Previous surveillance in live bird markets in Bali found that two out of seven markets, both from one district, had evidence of H5N1 virus (two positive out of 101 chicken and duck sampled) (unpublished data). These findings suggest that markets, where there is an intermingling of chickens and ducks from different sources have higher H5N1 activity in birds. Live bird markets are known to amplify and maintain avian influenza viruses.[11] Furthermore, H5N1 activity in markets is also a risk for human infection.[12–14] One example in Indonesia is in a highly remote part of West Java Province in August 2006, where infected poultry from the district market were introduced into the villagers' backyard flocks resulting in mass poultry deaths and three confirmed H5N1 human cases.[13] Furthermore, we found that the risk of likely H5N1 outbreaks increased in villages where birds are transported in and out of the village as part of commercial poultry production. The fact that the H5N1 virus is not uncommonly isolated from live bird markets provides ample opportunity for introduction of virus into household flocks through movement of persons, fomites or poultry. It was previously reported that opportunity for such activity was a risk factor for outbreaks of H5N1 in farms.[15] Our observations may indicate a similar risk in Bali and highlights potential strategic interventions that may help reduce transmission and maintenance of H5N1 virus within village poultry flocks.

Although pigs are commonly reared in close proximity to chickens and ducks in Bali and 65% of pigs sampled came from villages with H5N1 outbreaks, there was no evidence of H5N1 infection as judged by seroprevalence and

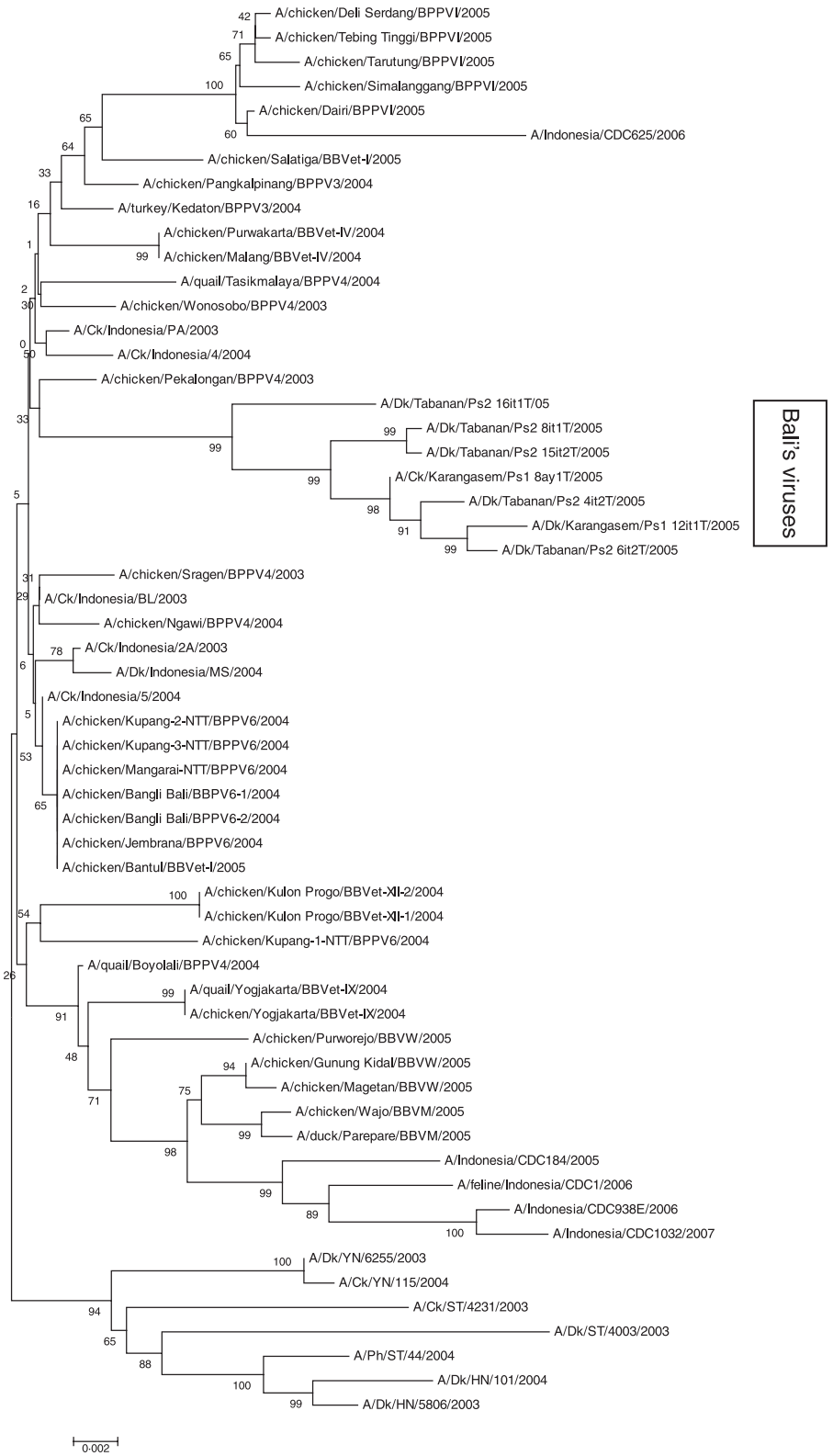

Figure 2. Phylogenetic tree of the haemagglutinin of the H5N1 viruses isolated from live bird markets. The region of the haemagglutinin from HA1 1–1570 have been analyzed using mega, version 3.1. A neighbor-joining bootstrap analysis (1000 replicates) using the Kimura two-parameter model is shown.

by virus isolation. Pigs experimentally infected with H5N1 virus shed virus but failed to transmit virus to uninfected littermates suggesting that this virus may not readily spread from pig to pig.[6] Seroepidemiological studies of pigs in Vietnam during the period of peak H5N1 outbreaks in poultry revealed very low level seropositivity (0·25%).[6]

The study showed increasing proportion of chickens with seropositivity with increased doses of H5N1 vaccine administration, up to a maximum seroprevalence of 60% in those reported vaccinated three times. It should be noted however that chickens are a dynamic population with turnover and chickens tested on the day of study may not necessarily have all received the three doses. The small number of chickens reportedly receiving over three doses of vaccine had an unexpectedly low H5N1 seroprevalence. However, all of these chickens were from one cluster (village). Further investigation is needed to determine whether the low seropositivity in this group could be attributed to systematic errors in vaccination in that village.

As expected, unvaccinated chickens had low H5N1 seroprevalence with only 2% of chickens seropositive. In contrast, 12 out of 57 unvaccinated ducks were seropositive (21%). This may reflect that ducks are more likely to survive natural infection. Alternatively, infection by low pathogenic H5 influenza viruses may contribute to this H5 seroprevalence since this may not be differentiated by the serological test. The prevalence of low pathogenic H5 viruses within ducks in Indonesia is unknown and needs further research.

Our study findings need to be interpreted in the context of several limitations which are common to other recent comparable studies.[8] In some cases, the definition for H5N1 outbreaks was based on a combination of serological and epidemiological factors rather than virological confirmation. Without confirmation of H5N1 virus, we are uncertain of the sensitivity and specificity of the definition. As there may have been variable time-intervals between local H5N1 outbreaks and the time of the study, serological responses may have decline to undetectable levels leading to a false-negative results. The turnover of livestock may also result in a similar effect. However, as serology did indicate seropositive chickens/ducks in villages with no vaccine history, this suggests that outbreaks did occur and were detectable within these constraints. Similarly, based on the surveillance conducted prior to our study period, over 50 H5N1 isolates were obtained from outbreak sites in all nine districts of Bali over the period of October 2004 to December 2005 (unpublished data), indicating that disease outbreaks continued up to the study period and there was potential for exposure. Indeed, H5N1 virus was readily detected in poultry markets during the study, but yet, market workers remained seronegative. These findings suggest that the conclusions we draw regards the low infection rates in pigs and humans are robust.

This study adds to the evidence that transmission of H5N1 to humans remains a rare event and exposure in itself is a necessary but not sufficient factor for explaining the occurrence of human disease. Our study also highlighted that the market chain and associated behaviors may play a role in the maintenance of the virus in household flocks. Further research into virus transmission pathways is needed to identify points of intervention that could cut transmission cycles and potentially reduce the risk for human infection.

Addendum

K Santhia, A Ramy, P Jayaningsih, G Samaan, A Putra, I Dibia and C Sulaimin designed the study. K Santhia, A Ramy, P Jayaningsih, G Samaan, A Putra, I Dibia, C Sulaimin, G Joni, C Leung, J Peiris analyzed the data. K Santhia, A Ramy, A Putra, T Wandra, G Samaan, J Peiris interpreted the findings and wrote the manuscript. I Kandun, E Tresnaningsih, A Putra reviewed the content of the study and provided final approval of the manuscript.

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