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計畫名稱：**跨越宿主傳播且具世界流行潛力的流行性感冒病毒整合偵測系統**

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註:請依契約書第九條之規定時程繳交，一式四份

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中文摘要

據世界衛生組織報導，**今(2002)年元月香港農場**自 1997 年流行之後已**再出現 H5 型禽流行性感冒病毒**(以下簡稱流感病毒)，雖經大量撲殺與隔離等措施，**十二月香港又可見 H5 型禽流感病毒蠢蠢欲動**，值得注意的是此型病毒的基因型的複雜度已不復 1997 年單純!因此，這些新型病毒是否以跨越宿主而感染人有待觀察，又在**近香港而人口密度過高的台灣如何及早建立此跨越宿主傳播的流行性感冒偵測系統是當前十分重要的公共衛生工作**。

本研究在過去的努力工作偏重：(1) 協助以最佳流行病學研究設計以明瞭台灣地區與離島家畜/家禽的流感病毒株與血清流行病學實況，並親赴金門實地探查聯絡，掌握其主要分佈以擇選合宜的跨越宿主傳播的「人群」流行病研究區：為宜蘭、彰化、屏東與金門；(2) 研究期間，發現宜蘭鴨群、桃園豬群、金門豬的血清流行病數據均顯示值得今年冬季密切追蹤偵測人流感病毒；(3) 已研展以反轉錄酵素一聚合酵素鏈反應 (reverse transcriptase-polymerase chain reaction，簡寫為 RT-PCR)**快速分子偵測台灣地區較多的家禽 H1，H5，H6,等非 H1-H3 病毒**，以備不時之需，並研討較經濟而有效的檢測法；(4)針對已有家畜/家禽疫情的地區，採集人喉頭檢體進行流感病毒偵測與分離。結果發現：(a) 比較 Madin- Daby Canine Kidney (MDCK)、雞胚與喉頭檢體三者的流感病毒分離率，其中以雞胚最佳(5/7=71.4%)，MDCK 的細胞培養法次之(5/10=50%)，而喉頭檢體(3/8=37.5%)，顯示在體外增殖病毒之後的檢測率較高；(b) 郊區小兒科採檢意願配合度極低，所幸仍分離得 A 型流感病毒；(c) 學童病假偵測在各區中，以金門十月底的病假缺席率較本島為高，惜電話多次，金門尚不願送檢體，現仍努力中；(d) 血清流行病學探究正已展開溝通、說服的抽血工作，也已親赴宜蘭討論實際做法，目前也已親赴宜蘭討論實際做法，目前十分需要農委會相關行政單位的全力配合。

綜言之，台灣有家畜、家禽郊區廣泛，本研究因經費有限，已嘗試串聯野鳥、豬、家禽、人的流感病毒偵測與學童病假偵測，並每月開會交換現況研討未來工作方向，如此**整合性流感病毒偵測可對病毒的演化、轉變為高毒性及流行變遷，有其公共衛生政策應用之實質意義**，才開始工作半年多，尚需研究團隊繼續努力。

ABSTRACT

Physicians participated in our flu surveillance system from February to June, 2002 obtained throat swabs from children presenting with flu-like illness. A total of 11 specimens were collected with tubes containing collection media and antibiotics on working days and sent directly to our laboratory by express mail. Each specimen was inoculated onto Madin-Darby canine kidney (MDCK) cells tissue culture for virus growth, performed hemagglutination tests, and examined their cytopathic effect (CPE). The positive samples showed CPE were re-inoculated into 10-day old embryonated eggs. For establishing a rapid molecular diagnosis for possible novel intra-species transmission flu A virus in Taiwan, we employed reverse transcriptase -polymerase chain reaction (RT-PCR) methods for detecting and subtyping influenza A viruses. Up to now, our RT-PCR system at NTU is capable to detect not only positive control of human H1, H3 but also avian H1, H5, and H6. Thirteen of total 25 samples tested by RT-PCR with avian H1 primer set showed positive results, including 3 in 8 throat swab (37.5%), 5 in 10 MDCK (50%), and 5 in 7 embryonated eggs fluid (71.4%). In summary, direct detection of flu A virus by RT-PCR may decrease the sensitivity of detection. In addition, MDCK cells provide the most convenience in amplifying virus. Future studies should try to inoculate chicken eggs for those samples without CPE from MDCK cells because the low passage of chicken eggs can provide virus stocks for vaccine preparation.

INTRODUCTION

The causing agents of influenza are influenza A, B and C viruses which belong to the Orthomyxoviridae family. Type A influenza viruses are the most important as they result in severe epidemics or pandemics and are responsible of important pathological outcomes. Subtypes of influenza A viruses depend on the nature of their surface glycoproteins: haemagglutinin (H) and neuraminidase (N). **Pandemic influenza due to constant changes of influenza A viruses** is a major public health problem with global concerns. **Three possible mechanisms are involved in antigenic variation. First**, the continuous "**antigenic drift**" due to point mutations in the gene coding for the H protein, leads to the progressive emergence of new viral strains and needs to annually evaluate the best composition of human flu vaccine in that indicated year. **Second, "antigenic shift"** due to genetic reassortment in pigs by which the gene coding for the H protein, or both genes coding for H and N proteins are involved, a new viral sub-type appears, then replaces the precedent and suddenly responsible human pandemics. **Third, the inter-species transmission** to facilitate flu A virus to jump across host species, the situation resulted in six deaths out of 18 patients in Hong Kong in 1997.

Ecology of Influenza involves the infection of many different hosts, including human beings and several animal species, such as ducks, chickens, geese, other domestic avians, wild birds, pigs and even ocean mammals. **Inter-species transmission of influenza A viruses has been documented to be related to several past flu pandemics.** In addition, several other findings support this potential human health threat. First, swine influenza viruses provide the best direct evidence for transmission of influenza viruses between species because **pigs are the best mixing vessels for different influenza A viruses.** Antigenically and genetically identical Hsw1N1 influenza viruses were isolated from pigs and man on the same farm in Wisconsin, U.S.A. Second, the isolation of H3N2 influenza viruses from a wide range of lower animals and birds suggests that **influenza viruses of man can spread to the lower orders**. Under certain conditions, the H3N2 viruses can even persist for several years in some species. Third, the isolation of a large number of influenza A viruses from **aquatic birds**, that possess surface proteins antigenically similar to the viruses isolated from man, pigs and horses, serves as **a reservoir of genetic pool or a role in the origin of some of the pandemic strains of influenza A** viruses through reassortment.

In December 1997, government workers in Hong Kong slaughtered 1.4 million

chickens to prevent the potential spreading of H5N1 across the globe (Tam, 2002). In addition, the reappearance of H5 in 2002 signifies the importance of inter-species transmission study in Taiwan. Therefore, the first year of this study is to establish integrated surveillance system of influenza virus and rapid laboratory methods to detect novel influenza A viruses which are not conventional human H1-H3.

MATERIALS and METHODS

1. Study Design and Areas

Since the rural areas are very large and impossible to do all those virological surveillance. This study select those areas with unusual outbreaks of flu in pigs, domestic avians and possible novel influenza A virus isolated from wild bird flu surveillance system. In other words, we contacted directly with the pediatricians located in those areas with unique flu virus isolated such as pig flu H1N2 in TaoYuan.

2. Study Subjects and Patient Specimens of Intra-species Transmission

During the spring influenza season in 2002, 11 throat swabs were collected from children according to the **case definition of flu-like illness** (fever, cough, sore-throat, and myalgia symptoms, with exclusion of mild rhinitis, bronchitis, tonsillitis by doctors' diagnosis) but a**dd one of the following three situations in the designed questionnaires** (fatal cases, animal contacted patents, patients or their family who ever been to Hong Kong, China, or southern asian countries within two months, and patients whose family or neighborhoods had flu-like illness cases). Specimens were obtained by each pediatrician participating in our flu active surveillance scheme form northern (Tao-Yoan General Hospital), central (Chang-Hua Christian Hospital), and southern (Ping-Tung Christian Hospital) Taiwan island. The samples were taken and stored in viral collecting medium, thereafter transported directly to the laboratory at the Institute of Epidemiology, National Taiwan University.

3. RNA and Primers of Positive Controls (Avian and Human Controls)

The reference RNAs for human H1, H3, avian H1, H3, H5, H6, H7 and H9 were kindly provided by Professor Kao at the National Taiwan University Hospital (NTUH) in Taipei and Ms. Ming-Shiuh Lee at the Institute of Animal

Health at Dam-Shui.. Six identified cell culture supernatants of clinical specimens from NTUH were also kindly provided by Professor Kao and served as ”positive controls” for re-confirmation.

4. Nucleic acid Extraction

A 280ul aliquot of collecting medium and culture supernatant was extracted to isolate the virus genomic RNA. Nucleic acid extraction of throat swab specimen or cell culture supernatant was performed by using QIAamp Viral RNA Minikit (Qiagen). 100µl to 200µl sample was extracted following suggested manufacturer’s protocol.

For embryonated eggs allantoic fluid, RNA was also extracted with Trizol reagent following suggested manufacturer’s protocol. The RNA extraction of embryonated eggs was using Trizol regent (Invitrogen Life Technologies). Briefly, 0.3ml of allantoic fluid was mixed with 1ml of Trizol reagent. After mixing completely and being kept at room temperature for 15 min, the mixture was extracted with 0.1ml chloroform. After centrifugation at 14,000 rpm for 15 min at 4℃, the RNA in the aqueous layer was precipitated by adding 0.25ml isopropanol. The RNA precipitate was collected by centrifugation at 14,000rmp for 10 min at 4 ℃, washed by 75% ethanol and dissolved in 40µl of RNase-free water.

5. RT-PCR

A one-tube RT-PCR was performed in a mixture (25ul) by using SuperScript one step RT-PCR system (Invitrogen Iife technologies). The cDNA was synthesized for 30 min at 50℃ of type A detection, or for 1 hour at 46℃ of type B and human H1+H3 detection. The PCR conditions of type A detection were 40 cycles of denaturing for 1 min at 94℃, annealing for 3 min at 55℃, and extension for 3 min at 72℃, followed by final extension for 10 min at 72℃. The condition of type B and human H1+H3 were the same as above, expect that the annealing time and temperature were reduced to 2 min at 53℃. Two sets of primers were synthesized to detect influenza A and B viruses from multiple species

primer set A: M52: 5’-CTTCTAACCGAGGTCGAAACG-3’, and
M253 5’-AGGGCATTTTGGACAAAKCGTCTA-3’;
primer set B: B55 5-ACAAATTGAGGTGGGTCCG-3’,and
B127 5’-ACCAGGGTAGTCAAGGGCT-3’).

The primers for human H1, H3, avian H1, H3, H5, H6, H7 and H9 were

kindly provided by Professor Kao and Ms. Ming-Shiuh Lee.

Considering of RNA degradation, we performed a **two-steps RT-PCR** for avian H1, H3, and H5 detection,. cDNA was synthesized by using SuperScript reverse transcriptase and random hexamers. Each 25ul reaction mixture contained 2ul of RNA, 5ul of 5× RT buffer, 500uM each deoxynucleoside triphosphate, 2.5uM random hexamer, and 0.4U of RNase inhibitor per ul (all from Invitrogen Iife technologies). After incubation for 10 min at 25℃, RT was carried out for 50 min at 42℃, followed by RT inactivation for 15 min at 70℃. The cDNA was stored at -20℃ before further use.

Only 10% of first-strand cDNA was used for PCR. The PCR conditions of amplification were just the same as we mentioned above.

A PE 9600 Thermocycler (Perkin-Elmer) was used to carry out PCR reaction. PCR products were electrophoresized and visualized on a 1.5% ethidium bromide-stained agarose gel using UV illumination. A 100-bp marker (5ul) was used as a control for fragment lengths.

RESULTS

一、 初步成果

整合性的流行性感冒偵測以下列各偵測系統簡述近況。

（一）人流感病毒偵測

今年一月香港發生高病原性禽流行性感冒病毒，致兩個雞場雞隻嚴重死亡，甚至香港政府下令撲殺上百個雞場的雞隻，加上 1997 年數百萬雞隻的大肆快速撲殺，造成經濟上極大的損失。今年本研究目標為：取得重要病毒以協助未來疫苗在台灣的開發,明瞭跨越宿主傳播的傳染機轉為何?在人能分離出非 H1、非 H3 的亞型；尤其是 H5、H9、H7、H6，未來可供跨越宿主傳播至人的傳染率為何？因此本計劃將目標為**偵測**流行性感冒病毒跨越宿主的傳播途徑。目前對於「流行季節」的定義，建議可把現況歸為流行季節前期，當數值超過基準值時，便可列為流行季了。今年在人的流感方面，Ａ型流感病毒在 2002 年有兩尖峰（一　二月及六　七月，七月大於六月），仍以 H1 和 H3 為主，且**以 H3 為多**，其中較引人注意的是有 5 株為**(四川)/99，**又臺大醫院有病例是來自同一家庭而有姊妹關係，可考慮調查此病例是否甫由大陸入境。今年５月多Ｂ型及Ａ型Ｈ$_3$。現尚未見非Ｈ１也非Ｈ３的流感病毒。金門、宜蘭檢體少，尚需加強，其他來自郊區的流感病毒在暑期分離不易，在 12 月正加緊努力中。

（二）學童缺席偵測

設計網頁讓校護上網填寫，而學校的設點，可限定於爆發流行的養殖場附近學校、診所或醫院。如此流感病毒偵測功能更能發揮。可由設點學校的學童缺席人數建立起基準值，當流行數據超過此基準值時，才需登錄基本資料，如此一來也可讓校護更加瞭解學校內學生健康情形。已設計妥２頁**學校偵測疾病網路版表格**，正與衛生署疾管局合作，由其原表格版做法改為每週各校自登錄的網路版偵測做法，此外原疾管局北、中、南、東的２５所國小偵測網，在**九月將擴及１２５所**，經由衛生局負責推動。預計 11~12 月推出，要全部做好，一起上路。學童缺席偵測中可見到金門 10-11 月較多，惜校護採檢動員不易，而自知金門高中有流行時，聯繫多次仍無法取得採檢。

做法上:以班級級任老師為單位，再經校護與衛生單位偵測系統作橋樑管道，經由教育部推動，此學校偵測會效果更大。金門建議應被列為病毒高度暴露區，而醫院急診可在流行季時再列入考慮範圍。

（二）禽流感病毒

在**野鳥流感病毒偵測**方面，自元月到 2 月，候鳥遷徙已到了尾聲，尚無分離到任何禽流感病毒。今年 3 月在金門野鳥分離得 H10，是否會傳給豬尚不知。另金門在地八哥鳥當作寵物入台檢疫發現 **H3N8**，但其後未再分離到。野鳥來說，**尖峰月份與家禽不同**，南鳥北上來台所帶病毒較少，而北鳥南下的 9　11 月為帶病毒的高峰。

在**家禽流感**病情方面，**H6N1** 感染情形較為嚴重，2002 年家禽由血清學看抗體數據知悉多為流感病毒 A H6N1 型，且那時抗體效價也高，感染率也高。

例如彰化多數的產蛋雞場被分離出 H6N1。據 H6N1 抗體力價統計可看出家禽養殖場 H6N1 陽性率佔了 47%- 49%。**嘉義**有一火雞場，於今年二月間，曾分離出 **H7** 禽流感病毒，只是後續未再見到，會持續監測之工作。此外，鴨子今年有分離到 H3N8，**在宜蘭、彰化見 H5N2**（注意：非 H5N1），宜蘭的三個鴨場毗鄰，但是不同場主人，**宜蘭**鴨農場曾數度出現 **H5**，幸後續至今均為弱病毒株。有趣的是**一旦 H3N8 出現時，其角色會蓋過原 H5N2 或 H4**。值得注意的是此 H5N2 的 H 序列與香港 H5N1 的 H5 有所不同。此外，宜蘭三星鄉的 7 月樣本中分離到 H3 為主，但無 H5。目前，禽類尚未見令人憂心的 H2。未來在雞場的設點可在爆發流行的豬場附近選定雞場。而金門為檢疫上一大高危險或高暴露區，值得密切觀察。甚至必要時，到場了解畜牧養殖業感染情形。加上小三通開放，更需要密切注意輸進動物的種類檢疫的情形。

家禽市場為每月巡查一次，今年元月和二月,均無分離出禽流感病毒。在雞農和豬農身上未曾被採血測血清抗體，日後可安排對陽性場，或是當疫情發生時(當數值超過基準值)，對相關工作人員進行抽血檢驗，達到偵測病毒功能，以防擴大範圍散佈，導致流行。

（三）豬流感病毒

豬場流感疫情方面，自 90 年 11 月到 90 年 12 月間，來自肉品市場豬隻曾分離到 H1N1，但是在臨床症狀方面未有嚴重影響，除了在桃園縣觀音鄉，有一豬場，甚至有母豬因為感染流行性感冒病毒，不但造成死流產，甚至造成母豬死亡！已前往為該場工作人員採血。

(a) 血清流行病學顯示，豬流感病毒在台灣地區終年杓可測得，以在 12 月至元月最高，九月始升，十月更高，且多為 H1N1 佔 22.3%，(而 H3N2 少)，而 2 月即降，且典型(classical swine-like) 也低。

(b) 金門的典型似豬型流感病毒卻較多，且無似鳥型(avian-like)，至 3 月而仍可見。

1、台灣本島：2001 年元至 12 月間，自台灣北中南 17 個肉品市場每月取 10 隻豬血（共 2100 血樣本）。北、中、南三區共 6 個養豬場，每月 10 頭肉豬鼻腔黏膜分泌液。

（a）**各月血清流感病毒抗體陽性率大小順序依次為：**

元月＞12 月＞11 月＞10 月＞7—9 月＞餘月

（b）**台灣本島諸流感病毒型別變化：**

（1）**年元月**多為 **H_1N_1古典豬型**較多。

（2）2001 **年 7－12 月**為 **H_1N_1禽型**較多。

（3）即同年有H_1N_1轉型之趨變（由古典豬型轉成禽型）

（4）此與 2001 **年人也較多 H_1是相同趨勢。**

（5）**豬流感病毒分離數自去年 10 月上升**，今年四月下降。

2002 年 3 月在**桃園縣一豬場分離出 2 株 H_1N_2，**另四月自高縣鳳山及北縣的肉品市場各分離２株H_1N_2，至今已有**六株 H_1N_2，**來

源是似禽型？人型？古典豬型？尚待進一步探究。H_1N_2豬流感病毒在歐美均早有分離報告。

（c）地域分布：北中南檢體來自肉品市場而無大差別。

２．**金馬地區樣本**：自 2001 年 1－12 月分季採樣，5 鄉鎮選 15 豬場，每季有 10 肉豬＋10 母豬進行採血測抗體，另採集若干數量鼻腔分泌物，結果發現：

（1）**夏季**流感**病毒仍活躍**

（2）**金門的似人型流感病毒較高**，是否意謂人傳給豬的可能性較高。

（3）金門的 H_1N_1為古典豬型。

（4）2002 年初金門豬又是**人**型為多，但自 2 月即降。換言之，金門豬的流感病毒與本島豬流感病毒有很大的不同。

(a)夏季流感病毒可能是該年冬季流行之先鋒，但仍需證實。

(b)未來應對台大學生、急診、內科多做宣傳，分離病毒，如此每月分佈數據才更精確。

(四) 研展以反轉錄酵素一聚合酵素鏈反應(簡寫為 RT-PCR)的快速分子偵測

1. Positive control

By running RT-PCR with primer set A, each reference RNA was amplified a band of **244 bp** as we expected **(Fig. 1).**

Six samples from National Taiwan University Hospital (ID number: 1501, 1507, 1305, 1436, 1479, and 2338) were also subject to one-tube RT-PCR reaction. The size of PCR products with influenza B and human H1+H3 primers were also identified to be 108bp and 067pb separately **(Fig. 2)**.

For avian influenza, only H1, H5, and H6 have expected amplification by using two-steps RT-PCR **(Fig. 3)**.

2. Specimens

By using the avian primer set of H1, 1 out of 8 collection medium extractant, 5 out of 10 MDCK cell culture supernatant, and 5 out of 7 allantoic fluid of embryonated eggs had amplification products **(Fig. 4, 5, 6).**

We also performed avian H5 for embryonated eggs allantoic fluid. Fortunately, there was no amplification products appeared (data not show).

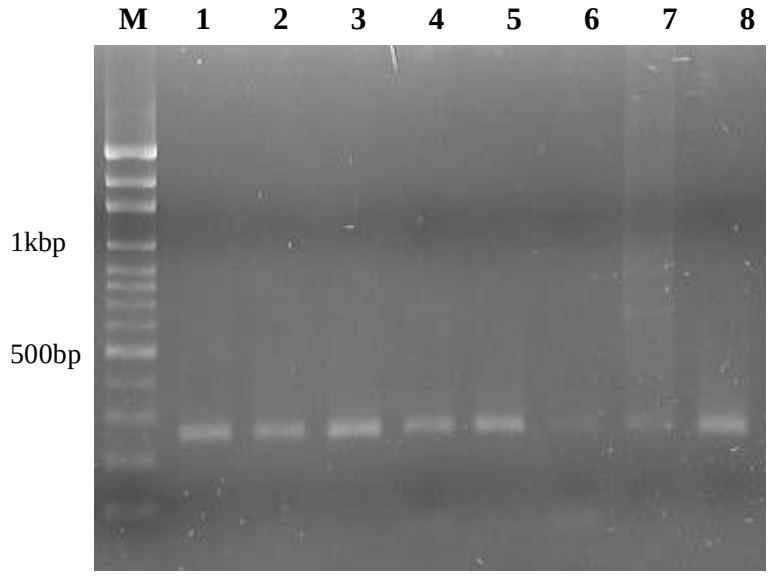

Fig. 1 HA typing of avian and human influenza RNA by RT-PCR. Each lane showed the typing result of a single reference RNA and indicated the products of expected size (244bp) for matrix gene of type A. Lane M: 100bp DNA size ladder maker. **Lanes 1-6: avian H1, H3, H5, H6, H7, H9. Lanes 7, 8: human H1, H3 RNA from NTU**.

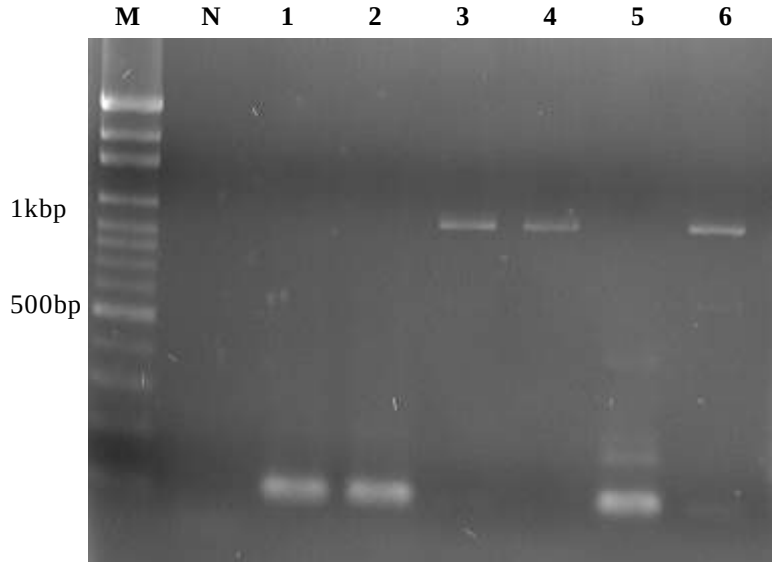

Fig. 2 Human B and H1+H3 typing of human influenza RNA by RT-PCR. Lane M: 100bp DNA size ladder maker. Lanes 1-6: 1501, 1507, 1365, 1436, 1479, 2338. Six identified clinical specimens from NTU. Each is represented by its registration number. RT-PCR products after amplification with primers specific to human B and H3 are 108bp and 967bp respectively. **Lanes 1, 2 ,5 are identified to be influenza B. Lanes 3, 4, 6 are identified to be influenza H3.**

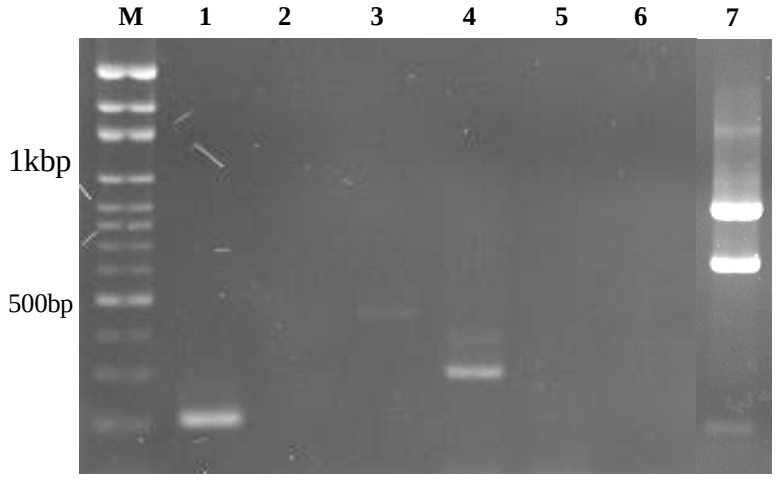

Fig. 3 The results of positive control RNA by two-steps RT-PCR. Lane M: 100bp DNA size ladder maker. **Lanes 1-6: avian H1, H3, H5, H6, H7, H9. for each subtype-specific avian primer sets**. **Lane 7: human H1+H3 samples detected by human H1+H3 mixed primer sets**. Only influenza of avian H1, H5, H6, and human H1+H3 had expected amplification of 200bp, 441bp, 325bp, 650bp, and 967bp respectively.

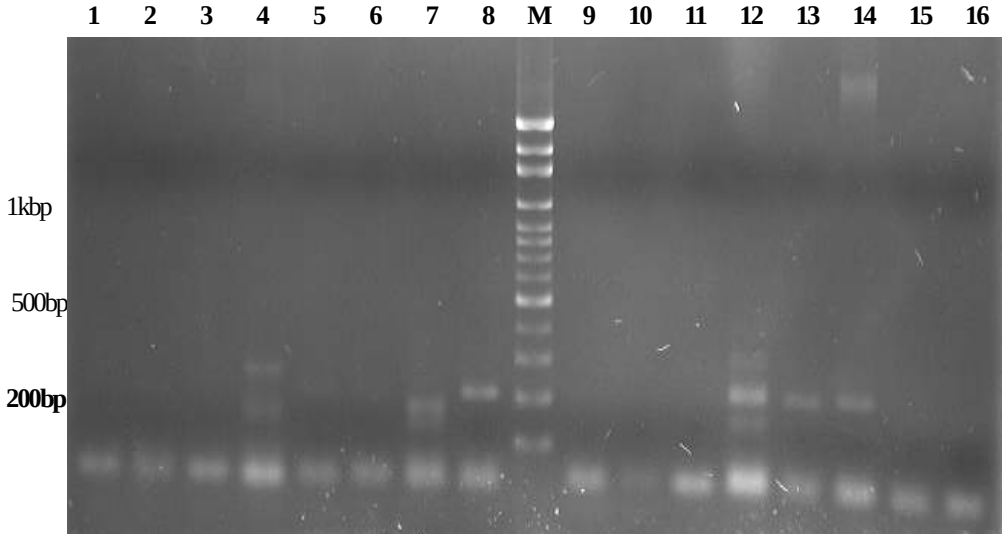

Fig. 4 The results of throat swab samples and cell culture supernatants by two-steps RT-PCR. The **primers** used are specific to **avian H1**. Lane M: 100bp DNA size ladder maker. **Lanes 1-8: throat swab samples (ID numbers of 104, 302, 303, 304, 305, 306, 401, and 402). Lane 9-16: MDCK cell culture supernatants (ID numbers of 14, 15, 16, 31, 32, 33, 34, and 35).** One clinical throat swab 402 (Lane 8:) and 3 MDCK culture supernatants (Lanes 12-14: 31, 32, and 33) were amplified. These products are agreed to the size of avian H1 amplification (200bp). Run the gel with similar sample no.

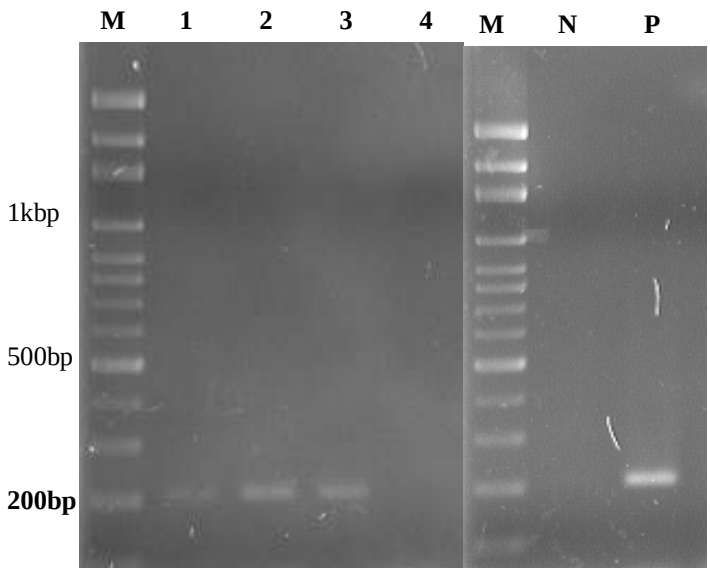

Fig. 5 The results of cell culture and embryonated eggs by two-steps RT-PCR. The primers used are specific to avian H1. Lane M: 100bp DNA size ladder maker. **Lanes 1, 2: MDCK** culture supernatants (ID #36, and #37). **Lane 3, 4: allantoic fluid of embryonated eggs** (ID #305, and #302). Two cell culture supernatants (Lane 1, 2: MDCK 36, 37) and one embryonated eggs (Lanes 3: egg 305) are amplified as the products with the size of avian H1 amplification (200bp). Lane N: negative control. Lane P: positive control.

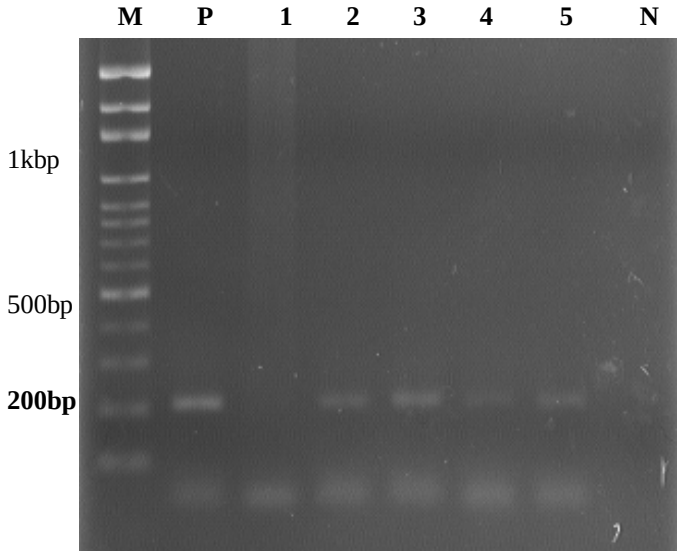

Fig. 6 The results of embryonated egg by running two-steps RT-PCR. The **primers** used are specific to **avian H1.** Lane M: 100bp DNA size ladder maker. **Lane 1-5: allantoic fluid of embryonated eggs** (ID #105, 301, 304, 305, and 309). Four embryonated eggs (Lanes 2-5: egg 301, 304, 305, and 309) are amplified as the products with the same size as avian H1 amplification (200bp). Lane N: negative control. Lane P: positive control.

DISCUSSION (討論)

1. What do we learn from the epidemic lesson in Hong Kong?

In 1997, a highly pathogenic avian H5N1 influenza virus was transmitted directly from live commercial poultry to humans in Hong Kong. The virus not only infected human but also caused high case fatality rate (30%) with six fatal cases out of the 18 infected persons identified. Mice study found that the molecular basis for the high virulence of this virus involved an **amino acid change in the PB2 protein**. Therefore, it is very important to do molecular epidemiologic study by sequencing all eight segments of novel influenza A virus, particularly the internal gene and HA.

2. Appearance of H9N2 in Human Populations

To eliminate the source of the pathogenic virus, all birds in the Hong Kong markets were slaughtered. In 1999, another avian influenza virus of H9N2 subtype was transmitted to two children in Hong Kong. In 2000-2002, H5N1 avian viruses reappeared in the poultry markets of Hong Kong, although they have not infected humans. Continued circulation of H5N1 and other avian viruses in Hong Kong raises the possibility of future human influenza outbreaks. Moreover, the acquisition of properties of human viruses by the avian viruses currently circulating in southeast China might result in a pandemic. Several Molecular epidemiologic studies of H9N2 found one isolate from quail in 1999 (Qa/HK/A/7/99) had high homology to the flu virus isolated from Hong Kong in 1997 [HK/156/97 (H5N1)] but another H9N2 Ck/HK/Fy20/99 strain had more similarities to chicken/duck isolates, indicating the multiple lineage of H9N2 already occurred in avian populations in Hong Kong.

3. Establishment of Rapid Molecular Diagnosis

Because of gradually setting up our assay procedure by using narrow budget, we firstly used reference RNA and primers to perform RT-PCR reaction. We also tested avian influenza H1 and H5 primer sets on samples (Fig. 4, 5, 6). Without further sequencing analysis, the positive PCR products by using avian H1 primers are suspected to be avian-like human H1 influenza. Although the primer sets which specific to type A and human H1+H3 had been tested by one-step RT-PCR on all clinical specimens, and cell culture fluid, no amplification has been attained (data not show) that might be caused by RNA degradation. We were testing on two-steps

RT-PCR with new protocol and adjustment, hopefully it will improve our diagnosis methods in the near further.

4. 實驗診斷法精益求精

今（2002）年美國喬治亞州已由 M 基因延展出測試禽流感病毒 H_5 與 H_7 型的快速分子檢測法（real-time reverse transcriptance PCR, RRT-PCR），此法比原養蛋法更便捷而敏感[Spackman etal., 2002]，值得注意的是他們所採用 H_5 與 H_7 的基因序列是參考北美洲的禽流感病毒，所以台灣必須按我們自己亞洲區所分離得的禽流感病毒序列來設計，畢竟飛亞洲的鳥與北美洲的不同！然而，本研究發現以禽的 H1 primer 可以 PCR 分子檢測來自禽的 HA 流感病毒 H1, H3, H5, H6, H7。由於經費考量，可先以培養法增殖流感病毒之後，再以免疫螢光法鑑定，以排除 H1, H3，所剩檢體若為陰性，再度培養以得病毒株，並同時以分子快速檢測為最妥。

5. 跨越宿主傳播

台灣在流感病毒跨越宿主傳播上，可有下列幾種可能：（1）豬流感病毒傳至人；（2）野鳥病毒傳至人；（3）家禽流感病毒傳至人；（4）野鳥先傳至家禽混種後，再傳給人；（5）禽流感病毒先傳至豬後，再傳給人；（6）人流感病毒先傳至豬而生新混種後，再傳給人。究竟哪一種機率較高，尚為未知數！事實上，在中國大陸動物與人住處較近，且不同動物在住家後院混養的情形也極為普遍，如此 1997-1982 及 1998 年在中國南方的豬身上均可測得 H_4 與 H_5 禽流感病毒，且 1998 年還測得 H_9 這種會感染人的禽流感病毒的抗體，其後也陸續在人與豬分離得 H_9N_2 病毒[Ninomiya etal., 2002]，顯示**確實可在華人身上發現由禽→豬→人的一連串感染**。所幸，台灣禽/豬混養處不多，但我們仍需努力偵測，這種地方不好找，也已花上我們不少時間選擇這些偵測點。

6. H_1N_2 型豬流感病毒

在病毒分離上，1998-99 年冬是以似人型的 H_3N_2 亞型為主，至 1999-2000 年及 2000-2001 年冬卻轉為以典型豬 H_1N_1 亞型為主，有趣的是自 2001 年 3 月起，似禽的 H_1N_1 亞型卻增，並於 2001 至 2002 年冬已取代原傳統豬型 H_1N_1 亞型，而成為主要流行型別。在血清流行病學上，豬流感病毒抗體在寒冷月份較高（32-75%陽性率），但春夏季較低（12-19%），值得注意的是病毒檢出率也是類似情形，且北、中、南各區並無地域差別而廣佈本島。

今年 3-7 月首次在台灣肉品市場及豬場共分離得 9 株豬群 H_1N_2 新亞型

流感病毒，包括 3 月份 2 株，4 月份 4 株，5 月份 2 株及 6 月份 1 株；其地理分佈為桃園縣 2 株，台南縣 2 株，台北縣 2 株，彰化縣 1 株，嘉義縣 1 株及屏東縣 1 株。目前尚待進行核酸序列比對分析，以追蹤其個別基因來源與性質，尤其是否可傳染人，值得探究，但已知一株桃園 H1N2 豬流感病毒的 HA 是來自禽類，顯示在台灣的流感病毒已可由禽傳給豬的實況。

此型在 1978-80 年及 1989-92 年日本豬群，即發現是由典型豬 H_1N_1 與似人的 H_3N_2 重組型，接著在 1987-88 年法國豬群，又見似禽 H_1N_1 與似人的 H_3N_2 重組型，英國 1994 年人的 H_1N_1 與人的 H_3N_2 重組型，此人的流感病毒傳至豬在近年愈來愈多，如 1994-98 年在法、義、歐洲均見人 H_1N_1 與似人 H_3N_2 重組型，再與似禽的流感病毒內部基因三度重組，美國 1999 年豬群是發現典型豬 H_1N_1 與 H_3N_2 三重組基因重組型，而比利時在 1999-2000 年也看到人 H_1N_1/似人 H_3N_2 再與典型豬 H_1N_1 三度重組型。換言之，豬的流感病毒之重新洗牌效率已大幅提高，其傳至人的危險性也相對提高，因此我們將在上述分離得 H_3N_2 的地區試採集人血進行深入研究。

綜言之，第一年計劃重點在培訓新血、尋找跨越宿主傳播偵測地點、研展非 H_1 又非 H_3 的分子快速檢測法及接洽最難採集的獸醫/畜牧場主與工作人員的血液，現仍要努力做好流行前的多項準備工作。

7. 2002 年禽流感監測情形

野鳥監測 1385 排糞樣本，只有在三月於金門分離到一株 H_{10} 病毒株；金門有許多鳥類輸送至台灣，由輸送檢疫鳥群咽拭採樣檢測流感病毒發現，576 檢測數中由畫眉鳥分離到 H_3N_8 病毒株；養雞場抗體監測由 ELISA 初篩檢抗體陽性的 1775 血清樣本中，確定有 249（27.7%）為 H_6 亞型抗體，而 H_5 及 H_9 抗體均為陰性；然而，台北縣某活禽市場監測鴨排遣檢體 150 樣本中，五月檢體分離到 H_5N_2 病毒株，該病毒經 HA 序列分析，不同於 1997 年香港的流感病毒株，對家禽也屬「弱」病毒株。回溯性追蹤鴨隻來自宜蘭三星鄉某鴨場，為免於病毒傳播至養雞場，造成病毒變異為強毒株之可能，該場被清場撲殺，消毒病停養。周圍其他養禽場目前仍持續監測中，幾個月內尚未再發現 H_5N_2 病毒；但至十一月左右，復出現 H5，**顯示台灣已有 H5 病毒登陸，且可能未完全消失，**顯示此禽流感病毒的主動偵測其擴及面異常重要。

RECOMMENDATION 建議

1. 「跨越宿主傳播」之流感病毒流行已為世界衛生組織密切注意觀察中。由於台灣進出香港與大陸人口近年增加，且香港曾發生嚴重的禽流感疫情，流感病毒易藉呼吸道傳染而帶入國內。因此建立台灣流行性感冒的病毒偵測網與快速實驗診斷已刻不容緩；同時**應告知國安局主動在中國大陸禽豬混養農場處**，積極採集相關檢體，以確切掌握新型流感病毒的出現及其擴散災情。

2. 本計劃已初步建立整合性的北部（桃園醫院)、中部（彰化醫院)、南部（屏東醫院)、東部（宜蘭縣醫院/診所）以及離島（金門醫院）的偵測網，並培育年輕鄉村地區防疫研究人才，以配合台灣未來社會急需和全球流感病毒偵測網的連結。因此，**建議衛生/農委會相關單位在地方上應互通疫訊**，目前仍停留在「密件」而致地方衛生局全然無法知曉。在此懇請農委會可與台大流行病學所合作，以確切明瞭鴨/豬農與畜牧人員的感染實況。

3. **金門馬祖地區**因小三通之故，**學童病假偵測網宜擴大學校數，並涵蓋至初/高中為妥。**

4. 在流行性感冒流行季時，**檢疫單位需出入境管理局電腦資料連線**，應載入出入境人員**住台灣何地、曾去大陸何處及回台灣何處**。

5. **郊區的人流感病毒偵測**地理範圍太廣，宜採參考文獻，以**小兒科肺炎病例，或有接觸動物/農場/旅遊中國大陸，或有多重器官衰竭，進行檢體採集。**

6. 政府與學術單位在通力合作，親自至未來可能流行的疫區，並及早**教導小兒科醫護人員、校護進行流感採檢與運送檢體工作。**

7. **農委會**主動聯絡湍地區獸醫/畜牧/養殖公會，**舉辦流行性感冒病毒流行前準備，並鼓勵獸醫相關人員一但遇農場疫情發生時，可早日參與抽血防疫工作。**

8. **動物疫情**必須及早訂定系統化的**預警性敏感度更高的指標**，避免發生類似 1997 年香港禽流感大量農場雞隻死亡情形。

9. 以分子生物學的方法偵測流感病毒，已為世界先進國家廣泛應用，台灣應進行更深入之研究，以提升品質。所以在快速實驗診斷流程上，宜考慮時效性及與流行病學數據的串連。

FUTURE DIRECTIONS

1. From molecular point of view, the specificities and sensitive of RT-PCR reactions will need to be improved.
2. The H5N1 “bird flu” incident in Hong Kong in 1997 was raised global attention of cross- species influenza infection. The additional requirement for rapid tests to detect viruses originating from no-human hosts is crucial in Taiwan. Therefore, we are going to meet the need by improving sensitivity and specificity on molecular diagnosis, and also pay more attention on surveillance scheme.
3. Because of genetic variation, the target regions in newly emerging strains complementary to the primers or probes used in an assay must be regularly analyzed by nucleotide sequencing to check for sequence mismatches between the primer or probe, and target sequences. In the event of sequence mismatches, the primers or probes should be updated in order to avoid false negative assay results.
4. To accumulate adequate data for analysis is the main subject on this study. Since the results may provide prediction and prevent avian influenza outbreaks in Taiwan.

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