COMPARATIVE ASSESSMENT OF AVIAN INFLUENZA VIRUS ISOLATION AND IDENTIFICATION USING REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION, EMBRYONATED EGGS AND CELL CULTURE

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MASTER OF VETERINARY PUBLIC HEALTH

CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN
SEPTEMBER 2007
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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY AND FREIE UNIVERSITÄT BERLIN IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY PUBLIC HEALTH

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THIS THESIS HAS BEEN APPROVED
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FOR THE DEGREE OF
MASTER OF VETERINARY PUBLIC HEALTH

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18 September 2007

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Mayuree Potima
ABSTRACT

The study was conducted to compare analytical sensitivity of three diagnostic methods and between two types of sample and to determine the minimum detectable of virus concentration of the assay for avian influenza virus isolation and identification. Cloacal swab and lung organ samples from specific pathogen free chickens were divided into three groups. The various concentrations of avian influenza virus were added to the suspension of samples. First analyzed by chorioallantoic sac inoculation of embryonated chicken eggs and alternatively, by haemagglutination (HA) and antigenic analysis (subtyping) by haemagglutination inhibition (HAI) using selected reference antisera Newcastle disease virus, then identification by using direct vet smart. Second method of analysis was done by inoculation on Madin Darby Canine Kidney (MDCK) cells and alternatively, by haemagglutination (HA) and antigenic analysis (subtyping) by haemagglutination
inhibition (HAI) using selected reference antisera Newcastle disease virus, then identification by using direct vet smart. Last method of identification use reverse transcriptase-polymerase chain reaction. The assay developed from this study indicates that all three methods are specific for the H5N1 influenza virus.

The virus isolation and identification by using embryonated chicken eggs and MDCK cells yield similar minimum detectable virus concentration. Virus isolation and identification by using RT-PCR had yield highest detectable virus concentration when compared with other diagnostic methods. Cloacal swabs and lung organs analysis result were not significantly different (p<0.05). This assay would be highly useful as a diagnostic tool to help identify of H5 Avian Influenza A virus isolate from poultry specimens. and control influenza epidemics.
ชื่อเรื่องวิทยานิพนธ์ การประเมินผลการแยกและจำแนกเชื้อไวรัสไข้หวัดนกโดยวิธีรีเวอร์ส-ทรานคริปเตซร่วมกับปฏิกิริยาลูกโซ่โพลิเมอร์สีกับปฏิกิริยาลูกโซ่โพลิเมอร์สีเหนือและเซลล์เพาะเลี้ยง

ผู้เขียน นางสาวมยุรีโพธิมา

ปริญญา สัตวแพทย์สาธารณสุขศาสตรมหาบัณฑิต

คณะกรรมการที่ปรึกษาวิทยานิพนธ์ ศ.ดร. สาทร ไมล์มัด  yanında ประธานกรรมการ (FU-Berlin)

บทคัดย่อ

การตรวจทางห้องปฏิบัติการทำโดยการแยกเชื้อโดยการฉีดไข้ฟิล์คหรือการใช้เนื้อเยื่อเซลล์เพาะเลี้ยงหรือการตรวจพันธุกรรมชนิดH5N1โดยวิธีรีเวอร์ส-ทรานคริปเตซร่วมกับปฏิกิริยาHaemagglutination (HA) และวิธีHaemagglutination Inhibition (HI) ร่วมกันการตกตะกอนในรูน (Agar gel immunodiffusion) จากการทดลองในการศึกษาเพื่อที่การประเมินเปรียบเทียบผลการแยกและจำแนกเชื้อไข้หวัดนกโดยวิธีรีเวอร์ส-ทรานคริปเตซร่วมกับปฏิกิริยาลูกโซ่โพลิเมอร์สีไข้ฟิล์คและเซลล์เพาะเลี้ยง โดยเซลล์ที่นำมาใช้เป็นเซลล์เพาะเลี้ยงชนิด Mardin Darby Canine Kidney cells (MDCK cells) แบ่งกลุ่มตัวอย่างที่ใช้ในการทดลอง 2 กลุ่ม จากการสวยงามตัวไก่ (cloacal swab) และปอด จากไก่ที่ปลอดจากโรค และใส่ไวรัสไข้หวัดนกแล้วละความเข้มข้นในกลุ่มตัวอย่าง โดยกลุ่มแรกทำการเพิ่มตัวอย่าง ด้านบนที่มั่นคงของไข้พิล์ค (chorioallantoic sac) และทำการตรวจหาไวรัส โดยวิธีการตกตะกอนด้วยเม็ดเลือดแดง เพื่อทำการแยกชนิดของเชื้อไวรัสโดยวิธีการยับยั้งการตกตะกอนโดยการทดสอบด้วยเอนติบอดีของโรค Newcastle หลังจากนั้นทำการตรวจแยกกลุ่มของโรก โดยวิธีการตรวจแยกกลุ่มโดยตรง (direct vet smart®) กลุ่มการทดลองที่สองทำการทดลองที่สอง ทำการตรวจโดยใส่กลุ่มตัวอย่างลงในเซลล์เพาะเลี้ยงชนิด Mardin Darby Canine Kidney cells และทำการตรวจหาไวรัส โดยวิธีการตกตะกอนด้วยเม็ดเลือดแดง เพื่อทำการแยกชนิดของเชื้อไวรัสโดยวิธีการยับยั้งการตกตะกอนโดยการทดสอบด้วยเอนติบอดีของโรค Newcastle หลังจากนั้นทำการตรวจ
แยกกลุ่มของโรค โดยวิธีการตรวจแอนติเจนโดยตรง (direct vet smart®) กลุ่มการทดลองที่สาม โดยทำการตรวจหาเชื้อไวรัสจากการสร้างผลที่ชัดเจนโดยวิธีตรวจแอนติเจนโดยตรง (direct vet smart®) กลุ่มการทดลองที่สาม

ผลจากการศึกษานี้จะพบวิธีการแยกและจำแนกเชื้อไวรัสไข้หวัดนกโดยการฉีดไข่ไก่และเซลล์เพาะเลี้ยง จะให้ผลในการตรวจที่คล้ายกันในการตรวจหาปริมาณไวรัสที่ความเข้มข้นสูงสุด วิธีการแยกและจำแนกเชื้อไวรัสไข้หวัดนกโดยการตรวจหาเชื้อไวรัสจากการสร้างผลที่ชัดเจนโดยวิธีการตรวจแอนติเจนโดยตรง (direct vet smart®) กลุ่มการทดลองที่สาม

การตรวจที่คล้ายกันอย่างมีนัยสำคัญทางสถิติ (p<0.05) จากการทดลองสามารถนำไปประยุกต์ใช้ในการวินิจฉัยโรคไข้หวัดนกสายพันธุ์ H5 จากตัวอย่างในเสื้อปีกและช่วยในการควบคุมโรคไข้หวัดนกได้
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<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>cDNA</td>
<td>compliment DNA</td>
</tr>
<tr>
<td>conc.</td>
<td>concentration</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Egg Infectious Dose 50</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>HA test</td>
<td>haemagglutination test</td>
</tr>
<tr>
<td>HI test</td>
<td>haemagglutination inhibition test</td>
</tr>
<tr>
<td>HPAI</td>
<td>high pathogenic avian influenza</td>
</tr>
<tr>
<td>ISO</td>
<td>International Standardization Organization</td>
</tr>
<tr>
<td>LPAI</td>
<td>low pathogenic avian influenza</td>
</tr>
<tr>
<td>MDCK</td>
<td>Mardin Darby Canine Kidney Cell</td>
</tr>
<tr>
<td>ml</td>
<td>milliter</td>
</tr>
<tr>
<td>min.</td>
<td>minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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sec  seconds
T     Threonine
VB    veronal buffer
WHO   World Health Organization
x  g   times the force of gravity
μl    microliter
## Influenza Genes and Their Function

<table>
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<tr>
<th>Gene</th>
<th>Segment</th>
<th>Size(nt)</th>
<th>Function</th>
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<tr>
<td>PB 2</td>
<td>1</td>
<td>2341</td>
<td>Transcriptase: cap binding</td>
</tr>
<tr>
<td>PB1</td>
<td>2</td>
<td>2341</td>
<td>Transcriptase: elongation</td>
</tr>
<tr>
<td>PA</td>
<td>3</td>
<td>2233</td>
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<td>HA</td>
<td>4</td>
<td>1778</td>
<td>Haemagglutinin: Host cell attachment</td>
</tr>
<tr>
<td>NP</td>
<td>5</td>
<td>1565</td>
<td>Nucleoprotein: RNA binding, part of transcriptase complex, nuclear cytoplasmic transport of vRNA</td>
</tr>
<tr>
<td>NA</td>
<td>6</td>
<td>1413</td>
<td>Neuraminidase: viral release</td>
</tr>
<tr>
<td>M</td>
<td>7</td>
<td>1027</td>
<td>Matrix: M1 major component of virion M2 integral membrane protein-ion channel</td>
</tr>
<tr>
<td>NS</td>
<td>8</td>
<td>890</td>
<td>Nonstructural: NS1 nucleus; effects on cellular RNA transport, splicing, translation. Anti-interferon protein</td>
</tr>
<tr>
<td>NS2</td>
<td></td>
<td></td>
<td>also known as NEP (nuclear export protein)</td>
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</table>
1. INTRODUCTION

1.1 Introduction

Influenza viruses are spherically or longitudinally shaped enveloped particles with an up to eight-fold segmented, single-stranded RNA genome of negative polarity. Influenza viruses belong to the Orthomyxoviridae family and are classified into types A, B or C based on antigenic differences of their nucleo- and matrix proteins. Avian influenza viruses (AIV) belong to type A. Excellent reviews on the structure and replication strategy of influenza viruses have been published recently (Sidoronko and Reichl, 2004).

The main antigenic determinants of influenza A virus are the haemagglutinin (H or HA) and the neuraminidase (N or NA) transmembrane glycoproteins, capable of eliciting subtype-specific and immune responses and protection.

On the basis of the antigenicity of these glycoprotein, influenza A viruses currently cluster into sixteen H (H1 - H16) and nine N (N1 - N9) subtypes. These clusters are substantiated when phylogenetically analysing the nucleotide and deduced amino acid sequences of the HA and NA genes, respectively (Fouchier, 2005).

The conventional nomenclature for influenza virus isolates requires implication of the influenza virus type, the host species (omitted in the case of human origin), the geographical site, serial number, and year of isolation.
1.2 Objectives of the study

1. To compare analytical sensitivity of three diagnostic methods for avian influenza virus isolation and/or detection

2. To compare the effect of re-isolation and detection on the sensitivity

3. To determine the minimum detectable of virus concentration of the assay

1.3 Significance of the study

1. Provide the scientific evidence of the validity of test result in a laboratory

2. Provide the estimate of the laboratory efficiency in detecting AI

3. Provide information necessary for the ISO 17025 application
2. LITERATURE REVIEW

2.1 Background of Avian Influenza virus

2.1.1 History of Avian Influenza virus

Avian influenza (AI) caused by influenza A viruses is a disease of many kinds of poultry, wild and caged birds characterized by marked variation in morbidity, mortality signs and lesions. In addition, the infection causes periodical epidemics in humans, pigs, seals, and a variety of birds (Swayne and Halvorson, 2003). AI viruses are members of the Orthomyxoviridae family. Within the family there are three types of influenza: A, B and C. Type B and C affect only humans (Scholtissek et al., 1983). The RNA virus is enveloped, sensitive to ether, chloroform and different chemical disinfectants. Influenza A viruses are divided, on the bases of the antigenic of the antigenic relationships in the surface glycoprotein’s haemagglutinin (HA) and neuraminidase (NA), into subtypes. There are at present 16 H subtypes and 9 N subtypes. The virus genome is single stranded, which multiplies in the same cell; progeny viruses may originate from the reassortment of parental genes derived from different viruses and theoretically 256 different combinations of progeny viruses may arise from two parental viruses (Murphy and Webster, 1996; Anon, 2000; Suarez, 2000).

In poultry the most virulent from of avian influenza was designated as fowl plague. In 1981, the term fowl plague was replaced with the term ‘highly virulent influenza virus infection. Recently the term Highly pathogenic avian influenza-HPAI base on the surface antigen and pathogenicity is suggested to be used (Anon, 2000). Currently , only viruses of H5 and H7 subtype have been shown to cause HPAI in
susceptible species, but not all H5 and H7 viruses are virulent. However, it has been proved that highly pathogenic avian influenza (HPAI) viruses emerge in domestic poultry from low pathogenicity (LPAI) progenitors of the H5 and H7 subtypes (Garcia et al. 1996; Senne et al. 1996; Perdue et al. 1997; Villarreal and Flores, 1998). Also, in 2004, highly pathogenic avian influenza was detected in commercial and backyard flocks in Thailand. Serious epidemics of HPAI have occurred in Thailand during 2004. The eradication of disease is the stamping out policy, by culling and destroying of birds. A total of about 50 million poultry have been killed with very high costs and financial losses to the community, and to the poultry industry. Recently several outbreaks of AI H5N1 have been confirmed among poultry in Cambodia, China, Hong Kong, Indonesia, Japan, Laos, South Korea, Vietnam and Thailand.

2.1.2 Taxonomy of Avian Influenza virus

Influenza viruses are enveloped single-stranded RNA viruses with a pleomorphic appearance, and an average diameter of 120 nm. Projections of haemagglutinin and neuraminidase cover the surface of the particle (Figure 1).
Figure 1: Structure of an influenza A virus. Image copyright by Dr. Markus Eickmann, Institute for Virology, Marburg, Germany. Used with permission. - http://www.biografix.de
The influenza A and B virus genomes consist of 8 separate segments covered by the nucleocapsid protein. Together these build the ribonucleoprotein (RNP), and each segment codes for a functionally important protein: Polymerase B2 protein (PB2). Polymerase B1 protein (PB1). Polymerase A protein (PA). Haemagglutinin (HA or H). Nucleocapsid protein (NP). Neuraminidase (NA or N). Matrix protein (M): M1 constructs the matrix; and in influenza A viruses only, M2 acts as an ion channel pump to lower or maintain the pH of the endosome. Non-structural protein (NS); the function of NS2 is nuclear export protein. The active RNA-RNA polymerase, which is responsible for replication and transcription, is formed from PB2, PB1 and PA. It has an endonuclease activity and is linked to the RNP. The NS1 and NS2 proteins have a regulatory function to promote the synthesis of viral components in the infected cell.

Avian Influenza Virus is a type A influenza virus. There are 16 subtypes of influenza virus known to infect birds. These subtypes are classified as “H” or “N”. Type A Influenza viruses can affect humans (H1N1, H1N2, H2N2), horses (H3N8, H7N7), pigs and many bird species. There are two forms of the type A Influenza viruses that affect birds, Low Pathogenic Avian Influenza (LPAI) and High Pathogenic Avian Influenza (HPAI). Avian influenza outbreaks in Asia have resulted in the death and depopulation of millions of birds and fatal disease in people with direct contact with infected birds.

2.1.3 Morphology and Nature of the Genome

Influenza particles are highly pleiomorphic: The morphological characteristics of influenza viruses are a genetic trait, but spherical morphology depends on passage in eggs or tissue culture (Subbarao et al., 1998). The majority of viruses are found to be
spherical, 80-120 nm in diameter, but many forms occur, including filamentous particles up to 2000 nm long. Different strains of virus tend to have different filamentous forms, a property which maps to the matrix protein, which lines the inside of the viral envelope (Klenk, 1974).

2.2 Avian Influenza virus

2.2.1 Epidemiology of Highly pathogenic AI of subtypes H5 and H7

Up to the end of 2003, HPAI was considered a rare disease in poultry. Since 1959, only 24 primary outbreaks had been reported world-wide. The majority occurred in Europe and the Americas. Most outbreaks were geographically limited, with only five resulting in significant spread to numerous farms, and only one which spread internationally. None of the outbreaks had ever approached the size of the Asian outbreaks of H5N1 in 2004 (WHO 2004/03/02). To date, all outbreaks of the highly pathogenic form have been caused by influenza A viruses of the subtypes H5 and H7. The original H5N1 virus, encountered for the first time in 1997, was of a reassortant parentage, including at least a H5N1 virus from domestic geese (A/goose/Guangdong/1/96, donating the HA) and a H6N1 virus, probably from teals (A/teal/Hong Kong/W312/97, donating the NA and the segments for the internal proteins), which underwent many more cycles of reassortment with other unknown avian influenza viruses (Xu, 1999, Hoffmann, 2000, Guan, 2002b). Several different genotypes of the H5N1 lineage have been described (Cauthen, 2000, Guan, 2002, 2003). The so-called genotype 'Z' has dominated the outbreaks since December 2003 (Li, 2004). A new dimension of HPAI outbreaks became evident late in 2003. From mid-December 2003 through to early February 2004, outbreaks in poultry caused by the Asian lineage HPAI H5N1 virus were reported in the Republic of Korea, Vietnam,
Japan, Thailand, Cambodia, Lao People's Democratic Republic, Indonesia, and China. The simultaneous occurrence in several countries of large epidemics of highly pathogenic H5N1 influenza in domestic poultry is unprecedented. All efforts aimed at the containment of the disease have failed so far. Despite the culling and the pre-emptive destruction of some 150 million birds, H5N1 is now considered endemic in many parts of Indonesia and Vietnam and in some parts of Cambodia, China, Thailand, and possibly also the Laos.

In January 2004, an outbreak of highly pathogenic avian influenza outbreak occurred in Thailand. HPAI was detected in a layer farm, Bangplama district, Suphanburi province, central region of Thailand. The peak of infection rate and seasonal influenza outbreaks was during the end of rainy season and winter season—it occurred in commercial poultry, backyard flocks.

2.2.2 Mode of infection and transmission

The disease can be transmitted horizontally by direct contact with infected birds or indirectly through contaminated equipments. There is little or no evidence of vertical transmission (egg-borne infection). However, eggshell surfaces can be contaminated with the virus (Cappucci et al., 1985; Tanyi and Klaczinski, 1992). Wild and domesticated waterflow are the major natural reservoir of influenza viruses. Representatives of all of the different subtypes of avian influenza A virus have been isolated from birds, particularly from aquatic species such as ducks, geese, and gulls (Hinshaw et al., 1981; Alexander, 2000). They may be infected with more than one subtypes without any clinical signs, excrete the virus for a long period and mostly do not develop detectable antibodies. A marked similarity between the subtypes’ prevalence in the waterfowl population and poultry were reported several times (Bahl et al., 1979; Halvorson et al., 1983, 1987). The infection can also be spread by contaminated shoes, clothing, crates, egg flats, and egg-case vehicles. The major way of the further spread of avian influenza viruses seems to be mechanical
transfer of infective feces (Utterback, 1984) through movement of man and contaminated equipment (Halvorson et al., 1980; Alexander and Spackman, 1981). The virus can survive in the contaminated environment for long periods of time at moderate temperatures and longer in frozen materials. Rodents and insects may mechanically carry the virus from infected to susceptible poultry.

Wild birds such as geese, ducks and game birds – they can be carriers of infectious agents shedding the virus in their feces without clinical signs of disease.

2.2.3 Clinical signs and Gross lesions

The severity of clinical signs, course and mortality in poultry after infection with avian influenza are extremely variable from highly acute to a very mild, or even inapparent form with few or no clinical signs and are influenced by many factor such as the virulence of the virus, the species, age of host, the immune status, concurrent diseases and management. Clinical signs include ruffled feathers, depression, diarrhea, a sudden drop egg production, cyanosis of combs, and wattles oedema, and swelling of the head, blood-tinged discharge from nostrils, respiratory distress, incoordination and pinpoint hemorrhages mostly seen on the feet and shanks. Lesions include swelling of the face, straw-color fluid in the subcutaneous tissues. Blood vessels are usually engorged. Hemorrhage may be seen in the trachea, proventriculus and throughout the intestines. Young broilers may show signs of severe dehydration with other lesions. In turkeys, lesions consist of sinusitis, tracheitis, pericarditis, petechial hemorrhages in pericardial fat, fibrinous airsacculitis, lung congestion, pneumonia as well as enlargement of the spleen and inflammation of the pancreas (Tanyi and Klaczinski, 1992; Swayne and Harvoxon, 2003).
2.2.4 Avian influenza infections in Humans

In humans, outbreaks of influenza A of subtypes H1N1, H1N2 and H3N2, appear to be the most common ones. Investigations showed that the most of human and mammal influenza viruses originated from avian sources (Webster et al., 1992). However Avian influenza A viruses do not usually infect humans (Swayne, 2000). In some cases, people with intensive contact with poultry and swine can be infected with other subtypes (Ito et al., 1998, Schotissek, 1994). Several instances of human infection and outbreaks have been reported since 1996.

In 1996 an H7N7 virus was isolated in England from the eye of the woman with conjunctivitis who kept ducks. This virus was shown to be genetically related to a virus of H7N7 subtype isolated from turkeys in Ireland in 1995 (Banks et al., 1998). Influenza A H5N1 was isolated from human in Hong Kong in 1997. This virus was identical with the HPAI H5N1 circulated in poultry (Class et al., 1998; Suarez et al., 1998). Again in Hong Kong in 1997, there were 18 confirmed human cases with 6 deaths. At this time, there are still no definite sign of human to human transmission, but even if it occurs, the efficiency of transmission is low at this time (Shortridge et al., 2000; Buxton Bridges et al., 2000). During the recent ongoing outbreaks in Asia with AI H5N1 from 2003 until 2007 (update 20 August, source World Health Organization), Vietnam has reported 95 confirmed cases in human and died 42 cases died.

Indonesia has report 102 which 81 have died. China has report 25, of which have 16 died. In Thailand has reported 25 confirmed case, of which 17 have been fatal. At this time it is believed that this case resulted from contact with infected birds or surfaces contaminated with excretion from infected birds. An investigation is ongoing to determine the source of human infection. After an incubation period of 1-3 days, the virus causes influenza-like symptoms characterized by high fever, chill,
headache, myalgia, prostration, sore throat, and cough. Children, elderly and those debilitated by chronic diseases or immunosuppression may be susceptible to infection and express severe manifestations such as difficulties in breathing and pneumonia. Risk populations includes poultry farmers, slaughterers, and handlers in contaminated areas. Avian influenza is different from human influenza, in which no transmission from person to person is evident.

2.3 Diagnosis of Avian influenza virus

2.3.1 Overview of Avian Influenza virus isolation

Although virus isolation remains the gold standard of diagnosis and is necessary for virus characterization, rapid laboratory confirmation of suspected influenza disease in routine diagnostic laboratories is usually performed by immunochromatographic or immunofluorescent detection of influenza virus antigens, or reverse transcriptase (RT). Regarding laboratory tests of avian influenza disease; many factors should be considered in deciding which tests to use. Sensitivity, specificity, turn-around-time, repeatability, ease of performance governmental regulation and costs should all be taken into account.

Clinical signs and lesions are not pathognomonic. Therefore, identification and the characterisation of the virus involved are essential.

The OIE subsequently adopted the following criteria for classifying an avian influenza virus as highly pathogenic:

a. Any influenza virus that is lethal for six, seven or eight of eight 4-8 week-old susceptible chicken within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid
b. The following additional test is required if the isolate kills from one to five chickens but is not of H5 or H7 subtype: growth of the virus in cell culture (for example primary cell such as chick embryo cells or cell lines such as MDCK cells, although most cell culture support the growth of HPAI influenza viruses or those of low pathogenicity in the presence of trypsin with cytopathic effect or plaque formation in the absence of trypsin) If no growth is observed, the isolate is not considered to be a HPAI isolate.

c. For all H5 and H7 viruses of low pathogenicity and for other influenza viruses, if growth observed in cell culture without trypsin, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be highly pathogenic.

According to the European Union legislation on avian influenza “Council Directive 92/40/EEC” the disease is defined as follows: avian influenza means an infection of poultry cause by any influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of haemagglutinin. (Hafez, H.M., 2005)

2.3.1.1 PCR detection

RT-PCR is generally more sensitive than serology and culture and the combination of RT-PCR with serology more sensitive than the combination of any other two methods (Zambon, 2001). RT-PCR can only be performed in well-equipped laboratory facilities by trained personnel. These methods can either detect both influenza A and B or differentiate between types (influenza A or B). The only direct
technique that has the potential to differentiate between subtypes (i.e. on the basis of haemagglutinin and neuraminidase) is RT-PCR.

2.3.1.2 Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR is a process whereby RNA is first converted to complementary DNA (cDNA) and a section of the genome is then amplified through the use of primers that bind specifically to this target area. This allows for exponential amplification of small amounts of nucleic acid, through the action of a thermo stable DNA polymerase enzyme, which enables highly sensitive detection of minute amounts of viral genome. Not only does RT-PCR have superior sensitivity (Steininger, 2002) but it can also be used to differentiate between subtypes and conduct phylogenetic analysis (Allwinn, 2002). RNA degradation of archival samples can decrease the sensitivity of RT-PCR (Frisbie, 2004). Therefore specimens should be processed as fast as possible after collection.

2.3.1.3 Other direct detection methods

Different methods exist for direct detection of influenza viruses. Some methods such as enzyme immunoassays (EIAs) can be suitable for bedside testing, others such as direct immunofluorescence allow for the preparation of slides onsite in clinics and the posting of fixed slides to a central laboratory (Allwinn, 2002).

2.3.1.4 Isolation method

Virus isolation or culture is a technique whereby a specimen is inoculated in a live culture system and the presence of live virus infection is then detected in this culture system. Since culture amplifies the amount of virus, it is more sensitive than direct methods, with the exception of RT-PCR. Virus isolation is only of use if the
live system or cells are sensitive for the virus that one intends to isolate. Isolation requires the rapid transport of specimens to the laboratory, since delays may lead to inactivation of virus (Allwinn, 2002).

2.3.1.5 Embryonated egg culture

Specimens are inoculated into the amniotic cavity of 9-11 day embryonated chicken eggs. High yields of virus can be harvested after 3 days of incubation (WHO, 2005). Since this technique requires a supply of fertilized chicken eggs and special incubators, it is no longer used for the routine diagnosis of influenza infection. However egg isolation provides high quantities of virus and is a very sensitive culture system. Reference laboratories therefore utilise this culture system to ensure high sensitivity and to enable the production of virus stocks for epidemiological monitoring.

2.3.1.6 Cell culture

Conventional culture: Various cell-lines are used to isolate influenza viruses, most commonly primary monkey kidney cells and Madin-Darby canine kidney (MDCK) cells. Some authors recommend the use of trypsin to aid virus entry into the cell lines (WHO, 2005). Conventional cell culture takes up to two weeks but has a very high sensitivity. Cytopathic effects such as syncytia and intracytoplasmic basophilic inclusion bodies are observed. The presence of influenza virus can be ascertained using haemadsorption using guinea pig red blood cells (Weinberg, 2005),
or immunofluorescence on cultured cells. The latter can also be used to type the isolated virus. Immunofluorescence has a higher sensitivity in detection of positive cultures than haemadsorption.

2.3.1.7 Real-time PCR

The principle of real-time PCR is based on monitoring of a fluorescent signal, which arises during the amplification process. Real-time PCR eliminates post-PCR processing of PCR products. This helps to increase throughput and reduces the chances of carryover contamination. The real-time PCR system is based on the detection and quantification of a fluorescent reporter (Heid et al., 1996). Taq Man probes are one of the main fluorescence monitoring systems for DNA amplification.

2.3.1.8 Serology

Serology refers to the detection of influenza virus-specific antibodies in serum (or other body fluids). Serology can either detect total antibodies or be class-specific (IgG, IgA, or IgM). Different serological techniques are available for influenza diagnosis: haemagglutination inhibition (HI), enzyme immunoassays (EIA) and indirect immunofluorescence. Serological diagnosis has little value in diagnosing acute influenza. In order to diagnose acute infection, an at least four-fold rise in titre needs to be demonstrate, which necessitates both an acute and a convalescent specimen. However it may have value in diagnosing recently infected patients. Serology is also used to determine the response to influenza vaccination (Prince, 2003).
2.3.1.8.1 Haemagglutination inhibition (HI)

HI assays are labour intensive and time-consuming assays that require several controls for standardization. However the assay reagents are cheap and widely available. Various red blood cells such as guinea pig, fowl and human blood group "O" erythrocytes are used. An 0.4-0.5% red blood cell dilution is generally used. Serum is pre-treated to remove non-specific haemagglutinins and inhibitors. A viral haemagglutinin preparation that produces visible haemagglutination (usually 4 haemagglutination units) is then pre-incubated with two-fold dilutions of the serum specimen. The lowest dilution of serum that inhibits haemagglutination is the HI titre. HI is has advantage that it is more specific in differentiating between HA subtypes (Julkunen, 1985).

2.3.1.8.2 Ezyme immuno assays (EIA)

EIAs are more sensitive than HI or CF assays (Bishai, 1978, Julkunen, 1985). Various commercial EIAs are available. but are not indicative of acute infection.

2.3.1.8.3 Indirect immunofluorescence

Indirect immunofluorescence is not commonly used as a method to detect influenza virus antibodies.

2.3.1.9 Rapid Tests

The clinical value of a diagnostic test for influenza is to a large extent dependent on the particular test's turnaround time. The first diagnostic tests that were developed for influenza diagnosis were virus isolation and serological assays. At that stage it took more than two weeks to exclude influenza infection. Although shell vial tests
have reduced the turn-around time of isolation, they are not generally regarded as rapid tests. The development of direct tests such as immunofluorescence enabled the diagnosis within a few hours (1 to 2 incubation and wash steps). Immunofluorescence tests however necessitate skilled laboratory workers and the availability of immunofluorescence microscopes.
3. MATERIALS AND METHODS

3.1 Location of the study area and data collection

This study as an experimental study using samples collected from pre-slaughter chicken proved to be free from avian influenza virus by isolation in embryonated chicken eggs and harvesting allantoic to detected haemaglutination test, then indirect test with Newcastle disease virus serum which free from disease at Animal Health and Technical Service in Bangkok. Cloacal swab and lung samples were collected from 100 chickens.

3.2 Highly pathogenic avian influenza virus (H5N1)

The avian influenza virus strain H5N1 (A/chicken-2 /NP/Thailand//2004) was used.

3.3 Sample size determination

30 cloacal swabs and 20 lungs were collected from 10 apparently healthy chickens per group. In this experiment, there were 10 set of 10 chicken each groups were examined. The factorial experimental design was used with 100 samples in each block of analysis method and each sample type (Table 1)
Table 1. Total number of experiment

<table>
<thead>
<tr>
<th>Virus conc.</th>
<th>Cloacal swabs</th>
<th>Lung organs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Embryonated eggs.</td>
<td>MDCK cell line</td>
</tr>
<tr>
<td></td>
<td>Embryonated eggs</td>
<td>MDCK cell line</td>
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<td>HA×10^0</td>
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</tr>
<tr>
<td>Total</td>
<td>100</td>
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</tr>
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</table>
3.4 Collection of the samples

3.4.1 Cloacal swabs

The cloacal swab of live pre-slaughter chickens were collected: using a dry cotton or polyester swab, it is inserted into the cloacal opening of live chickens and swabbed by inserting a swab deeply into the vent and vigorously swabbing the wall in place for a few seconds. Then it is slowly withdrawn with a rotating motion down the inside of the cloacal. The tip of the swab is put into a vial containing 30 ml of transport medium (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0.025mg/ml). Repeat as the first swab, 3 cloacal swab per one chicken. Each group had 10 chickens and then pool 30 cloacal swabs. After that, divided into three groups. First group for positive control. Second group for negative control and the last group for test by differential concentration of virus suspension.

3.4.2 Lung samples

The lung samples were collected from live pre-slaughter chicken which were sent to an Animal Health and Technical Service laboratory in Bangkok. where the carcasses were opened, then 20 lungs were collected from 10 chickens per sample and then the organs were pool as one sample and homogenized. After that, divided into three groups. First group for positive control. Second group for negative control and the last group for test by differential concentration of virus suspension.

The specimens for virus isolation were chilled in the ice pack immediately after collection. The cloacal swabs and tissue samples must be kept at 4ºC for inoculated of differential concentration of virus suspension.
3.5 Titrating Avian influenza H5N1 virus for infectivity

The aim of titration is to measure the concentration of infectious avian influenza disease virus in a suspension. The unit of measurement of infectivity of virulent avian influenza disease virus is the 50 percent Embryo Infectious Dose or EID50. To determine the infectivity titer of suspension of avian influenza disease virus, a series of ten-fold dilutions is carried out on the suspension. This data is used to calculate the inoculum.

Materials

Use specific pathogen free embryonated eggs, 9-11 days old. Candle the eggs and mark the inoculation site. 70% ethanol, needle 22 gauge, 1½ inch. syringe 1ml, egg hole punch, glue or varnish. 15 ml tubes & rack. 10 ml pipettes, forceps (sterile).

Methods

Step 1. Carry out ten-fold serial dilutions of the test suspension of virus.
   The range of dilutions required will be determined by the estimated infectivity titre of suspension in each 0.2 ml of inoculum.
Step 2. Estimate the infectivity titre in the suspension by considering previous titrations and storage conditions. dilute from 10⁻¹ to 10⁻⁹.
Step 3. Inoculate via allantoic sac 5 eggs with each dilution. Use separate needle and syringe for each dilution.
Step 4. Incubate eggs for 4 days at 38°C.
Step 5. After 4 days incubation, harvest allantoic fluid from each egg and test for hemagglutination to determine the presence or absence of Avian Influenza virus.
Step 6. Tabulate the results in the daybook.
Step 7. Perform the result of application of the Spearman-Karber formula to calculate the infectivity titre of the original suspension;
Log\textsubscript{10} Median Dose = \( (X_0 - (d/2) + d(\sum r_i/n_i)) \)

- \( X_0 = \log_{10} \) of the reciprocal of the lowest dilution at which all test inocula are positive.
- \( d = \log_{10} \) of the dilution factor (i.e. the difference between the log dilution intervals)
- \( n_i = \) number of test inoculated used at each individual dilution (after discounting accidental losses)
- \( \sum (r_i/n_i) = \sum (P) = \) sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.

\[
\text{EID}_{50} = 8.0 + 1/2(1.0) - 1.0(1+4) / 5
= 8.0 + 0.5 - 1.0
= 7.5
= 10^{7.5} \text{ per 0.2 ml.}
\]

The total amount of virus \( 10^{7.5} \text{ EID}_{50/0.2\text{ml}} \)

### 3.6 Preparation of serial ten fold dilution of virus inoculums

The stock virus concentration of avian influenza virus antigen (EID 50) was \( 10^{7.5} \text{ EID}_{50/0.2\text{ml}} \). Making ten-fold serial dilutions in PBS. The serial dilution of virus concentration were prepared for inoculated into preparation of sample.

Step 1. Set up the sterilized glass test tubes in a rack. Label each tube clearly to indicate the dilution of its content after the ten-fold serial dilution has been carried out.

Step 2. Use a micropipette to dispense 2.7 ml of the diluent (PBS 1x, pH 7.2) to all the labeled sterile tube.

Step 3. Use a micropipette to transfer 0.3 ml of the suspension of virus to the first tube and mix. This is the first tube of the ten-fold dilution.
Step 4. Use a micropipette with new sterile tip to carry out a second ten fold dilution. Step 5. Continue the series of ten-fold dilutions until the last tube.

The virus concentration from ten-fold dilution into each tube and all dilution of virus inoculums were keep at -70 °C. (Prepare for inoculation into the experimental samples)

3.7 Laboratory diagnosis methods

Laboratory procedures: The study was be conducted following the procedure by the Animal Health and Technical Service Laboratory in Bangkok for avian influenza virus isolation and identification using specific pathogen free embryonated egg (Bangkok Agro. Co, Ltd.) and MDCK cell line (CMU. Lab.) for virus diagnosis. This procedure allows virus identification by Haemagglutination test, Haemagglutination inhibition test by using Newcastle disease virus serum and RT-PCR.

3.7.1 Virus isolation and identification

3.7.1.1 Processing materials for virus isolation

3.7.1.1.1 Cloacal swab

30 cloacal swabs collected will be pooled as one sample was suspended in buffer containing antibiotics 30ml (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0.025mg/ml). Take the cloacal swab out before inoculation. After that, the positive control in first group inoculated 200 μl avian influenza virus HA $10^{7.5}$ . Second group inoculated 200 μl PBS 1x for negative control. Last group inoculated 200 μl for initial suspension of virus concentration. The
suspension form 3 group were centrifuged at 3000 for 30 min in refrigerated centrifuge (4°C) to sediment tissue debris and most bacteria, the topper clear liquid was collected, then the liquid was filtrated with filter membrane (0.45μm). For positive control group take the inoculated material 3ml into the microcentrifuge tube, the second group was negative control take the inoculated material 3ml into microcentrifuge tube, and the last group for initial suspension of virus concentration split into 10 duplicate.

For the next dilution (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹) same procedure as mention above was used.

3.7.1.1.2 Tissue samples (lung organs)

20 lung samples collected from 10 chickens will be pooled as one sample for virus isolation. Grind tissue in a sterile mortar and pestle with crushed glass from a pasteur pipette, making a 10% suspension with transport medium was suspended in buffer containing antibiotics 30 ml (isotonic PBS, pH 7.2, penicillin 2000 IU/ml, streptomycin 2mg/ml and fungizone 0.025mg/ml), then transfer homogenize sample with transport medium into centrifuge tube. For the positive control in first group inoculated 200 μl avian influenza virus HA 10⁷.5. Second group inoculated 200 μl PBS 1x for negative control. Last group inoculated 200 μl for initial suspension of virus concentration. The suspension form 3 group were centrifuged at 3000 for 30 min in refrigerated centrifuge (4°C) to sediment tissue debris and most bacteria, the topper clear liquid was collected, then the liquid was filtrated with filter membrane (0.45 μm). After that the positive control group take the inoculated material 3ml into the microcentrifuge tube, the second group was negative control take the inoculated material 3ml into microcentrifuge tube, and the last group for initial suspension of virus concentration split into 10 duplicate.

For the next dilution (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹) the same procedure as mention above was used.
3.8 Virus isolation by inoculation in embryonated eggs

Materials

Use specific pathogen free embryonated eggs (SPF), 9-11 days old. egg candler. 70% ethanol. needle, 22 gauge, Syringe 1 ml. 1½ inch. egg hole punch. glue or varnish. inoculum. discard tray.

3.8.1 Candling of eggs

Examine eggs with an egg candler and place with blunt end up into egg trays. discard any eggs that are infertile, have cracks, are underdeveloped, or that appear to have a porous shell.

3.8.2 Inoculation of eggs

Place eggs with blunt end up into holding the egg up to the candler, locate the embryo. egg trays and label each egg with a specific identification number. Wipe the tops of the eggs with 70% ethanol and punch a small hole in the shell over the air sac. Three eggs per specimen are usually inoculated in the allantoic sac. Aspirate 200 µl of processed sample into a tuberculin syringe with a 22 gauge, 1½ inch needle. Insert the needle into the hole of the egg. Using a short stabbing motion, inoculate 200 µl of the process sample which is prepare from cloacal swab and lung organs in each dilution for virus concentration into allantoic cavity, 10 samples per dilution. Remove the needle. Inoculate the two other eggs in the same manner with the same syringe and needle for a total of three eggs inoculated per specimen. Discard syringe into a proper safety container. Seal the holes punched in the eggs with a drop of glue. Incubate the eggs at 38ºC to 39 ºC and humidity of 60%-70% for 5 days.
3.8.3 Observation of inoculated eggs

Post-inoculation egg were candled to 5 days. Record deaths and note the time it takes for the embryo to die. Deaths within the first day are regarded as non-specific and discounted. Discard these embryos. Deaths on subsequent days are likely to be due to avian influenza disease virus. (Figure 2)
Figure 2: Observation of inoculated eggs
3.8.4 Harvesting of inoculated chicken eggs

Eggs are chilled at +4°C overnight or for 4 hours before harvesting. Label one plastic tube (15ml) for each egg with the specimen number. Clean off the top of each egg with 70% ethanol. With sterile forceps, break the shell over the air sac and push aside the allantoic membrane with the forceps. Then using a syringe and needle, pierce the amniotic sac and remove as much amniotic fluid as possible. Place harvest in a separate tube, combine the allantoic fluid from the three eggs inoculated per specimen. Centrifuge harvested fluids and perform a hemagglutination test and incubate at 4°C/30 mins.

3.8.5 Haemagglutination test

Material

Use 96 wells microtiter plate with V-bottom shape, multichannel micropipette 5-50 µl, single micropipette 5-50 µl, microtip 1-200 µl, timer, phosphate buffer saline (PBS) pH 7.2, 1% chicken red blood cell, positive and Newcastle disease virus serum.

Method

Using the V-bottom disposable tray. Dispense 50 µl of allantoic fluid from each sample into a well of the microwell plate. Use a separate tip for each sample. Include negative and positive control allantoic fluid samples on one of the plates. Dispense 50 µl of PBS into the wells. Add 25 µl of 1 percent red blood cells to each well. Gently tap sides of the plate to mix. Place a cover on the plate. Allow the plate to stand for 45 minutes at room temperature. Observe and record the results. Read results.
Interpretation of the result

HA negative: A sharp button of red blood cells at the bottom of the V-bottom well.
HA positive: A hazy film of red blood cells, no button or a very small button of red blood cells at the bottom of the V-bottom well. This will be show complete haemagglutination and contains one haemagglutinating unit.

The positive result from hemagglutination test occur in the first passage, so harvest allantoic fluid and store at -70°C. For the negative result from haemaglutination test in the first passage and continuing inoculate allantoic fluid from first passage in second passage.

The positive sample from Haemagglutination test continuing to confirmed Haemagglutination inhibition test by using Newcastle disease virus (NDV) antiserum.

3.8.6 Haemagglutination Inhibition test. (HI identification)

Using V-bottom disposable tray. Dispense 25 μl of allantoic fluid from each sample into a well of the microwell plate. Use a separate tip for each sample. Include negative and positive control allantoic fluid samples on one of the plates. Add 25 μl of Newcastle disease virus (NDV) antiserum. Add 25 μl of 1% chicken red blood cell suspension to each well. Gently tap sides of the plate to mix. Place a cover on the plate. Allow the plate to stand for 45 minutes at room temperature. Observe and record the results.

Read results.

Interpretation of the result

HI negative: A sharp button of red blood cells at the bottom of the V-bottom well.
HI positive: A hazy film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V- bottom well. This will be show complete haemagglutination inhibition and contains one haemagglutinating inhibition unit.

If the Newcastle disease virus (NDV) Haemagglutination test is negative, then, confirmed by subtyping with direct rapid test by using vet smart® test.

3.8.7 Rapid test by using vet smart® test

The commercial test kit vet smart test ®. Rockeby Avian Influenza Virus Antigen was used.

The kit is a Avian Influenza virus antigen detection (Test Type A) for invitrogen diagnostic use, Rockeby biomed. Singapore. Pte. Ltd).

This assay is designed to measure the quality of antigen. The 200 μl of the samples were taken from each tube which suspected avian influenza result from hemagglutination inbition test and pool as one sample. Take a portion of the sample (atlantoic fluid) from microcentrifuge tube with the sample collection swab. Insert the swab into the specimen tube containing assay diluent. Mix the swab until the sample has been dissolved into the assay diluent. Leave the test tube until the large particles have settled down to the bottom of the tube. Remove the test device from the foil pouch, and place it on a flat and dry surface. Using the disposable dropper provided, take the supernatant from extracted sample in the tube. Add eight drops into the sample hole with disposable droper. (Figure 3). Interprete test results after 10 minutes.
Interpretation of the result;
C: control band is found only this band it is the negative result
T: test group is test the sample
Negative result from the test found C band
Positive result from the test found both of C and T band

The direct rapid test by using vet smart® test for the last step to detect antigen in the suspension of experimental study in each dilution of virus concentration. The positive result in rapid test use to performable record the result. (For virus isolation and detection by using embryonated eggs)
Figure 3: Interpretation of the result form allantoic fluid by using direct rapid vet smart® test
3.9 Virus isolation by inoculation on Madin-Darby Canine Kidney cells (MDCK)

Materials

Use Madin-Darby Canine Kidney cells (MDCK), American Type Culture Collection (CMU, Lab). T-75 and T-25 tissue culture flasks, canted neck. Corning Cat. 430720. Dulbecco’s Modified Eagle Medium (D-MEM), Bovine serum albumin fraction V, 7.5% solution, Fetal bovine serum, 40 nm filtered, Trypsin-EDTA.

3.9.1 Preparation of MDCK cells in tissue culture flasks

The procedure for preparing an MDCK cell suspension is described for confluent T-75 flasks. If cell culture flasks of other sizes are used, the volumes have to be adjusted accordingly. One T-75 flask with a confluent monolayer of MDCK cells contains approximately $10^7$ cells. Decant medium and add 5 ml of trypsin-EDTA pre-warmed to 37°C. Distribute trypsin-EDTA over entire cell sheet by gently rocking the flask for 1 min. Remove trypsin-EDTA with pipette. Add another 5 ml of trypsin-EDTA solution and rock flask as described above for 1 min. Remove trypsin-EDTA with pipette. Add 1 ml of trypsin-EDTA solution. Distribute trypsin-EDTA over entire cell sheet and incubate flask at 37°C until all cells detach from plastic surface (5 - 10 min). The flasks may need shaking or tapping to detach cells. Add 1 ml of FBS to inactivate remaining trypsin. Add 8 ml of complete D-MEM. Pipette up and down gently to break up cell clumps. Transfer the 10 ml mixture to 90 ml of complete D-MEM containing 10% FBS for a final concentration of 10% FBS. (This cell suspension contains approximately $10^5$ cells per ml.). The remaining cell suspension can be used to seed T-75 flasks for cell passage.
3.9.2 Inoculation of cell culture

Add 6 ml (600,000 cells) of this cell suspension to an appropriate number of T-25 flasks. Incubate flasks at 37°C in 5%CO₂ incubator 2 to 3 days.

3.9.3 Preparation of flasks

Check the cells with microscope at 40X magnification. Replace growth medium with medium for virus growth. Be sure to use proper media as indicated. Decant growth medium into a beaker and wash three times with 6 ml of D-MEM containing 2 µg/ml of TPCK-trypsin.

3.9.4 Inoculation of flasks

Remove D-MEM from flask with sterile pipette. Inoculate 200μl of processed sample of each specimen into a T-25 flask using sterile pipettes including positive and negative control. Allow inoculum to adsorb for 30 minutes at 37°C in 5%CO₂ incubator. After that add 6 ml of complete media (D-MEM) containing 2 µg/ml of TPCK trypsin without calf serum to T-25 flasks. Observe daily for cytopathogenic effect (CPE) to 5 days.

3.9.5 Harvesting of flasks

Harvest the cell culture if CPE occurred on cells and observed by collecting supernatant fluid and adding stabilizer such as bovine serum albumin to a final concentration of 0.5%. Harvest by day 6 or 7, even if no CPE is observed. Label one plastic tube (15ml) for each flask with the specimen number. Centrifuge harvested
fluids at 3000 for 5 min. in refrigerated centrifuge (4°C) to remove cells and perform a haemagglutination test and incubate at 4°C/30 min.

Interpretation by observation on MDCK cells

If there is CPE on the cell line occur post inoculation in the first passage. Then harvested the media to detect Haemagglutination test. For the negative result in the first passage and continuing inoculated in second passage.

The Cytopathogenic effect was manifested by cell rounding, cell shrinkage and foci of cell destruction. Floating into the supernatant.

3.9.6 Haemagglutination test

(See 3.8.5)

3.9.7 Haemagglutination Inhibition test (HI identification)

(See 3.8.6)

3.9.8 Rapid test by using vet smart® test

(See 3.8.7)
Figure 4: normal MDCK cells

Cytopathogenic effect on cells; cell shrinkage and manifested by cell rounding

Figure 5: Cytopathogenic effect on cells
Cytopathogenic effect on cells; foci of cell destruction and floating into the supernatant

**Figure 5:** Cytopathogenic effect on cells
3.10 Virus identification by RT-PCR

3.10.1 Ribonucleic acid Extraction (RNA Extraction)

The commercial test kit Viral Nucleic Acid Extraction kit® (RBC Real Biotech Corporation, Taiwan), was used.

Viral Nucleic acid Extraction kit is specially designed for purification of viral RNA/DNA from cell-free samples such as serum, plasma, body fluids and the supernatant of viral infected cell culture.

Viral RNAs were extracted using a Viral Nucleic Acid Extraction Kit from supernatant of lung organ and cloacal swab: Transfer 200µl sample (supernatant from lung organs and cloacal swabs) into a microcentrifuge tube (not provided) if sample volume is less than 200 µl with PBS (not provided). Add 400 µl of VB Buffer (carrier RNA added) to the sample, mix by vortexing. Incubate at room temperature for 10 minutes. Place a VB column in a 2ml collection tube. Add 400 µl of 70% ethanol to the sample lysate and mix immediately by vortexing. Apply 600 µl of ethanol-added mixture from previous step to the VB column. Centrifuge at 6,000 xg (8,000 rpm) for 1 minute. Discard the flow-through and apply any remaining mixture from step 1 to the same VB column. Centrifuge at 6,000 xg (8,000 rpm ) for 1 minute. Discard the collection tube containing the flow-through and transfer the VB column to a new 2 ml Collection Tube. Add 400 µl of Wash 1 buffer into the VB column. Centrifuge at 6,000 xg for 30 seconds. Discard the flow–through and place the VB column back in the collection tube. Add 600 µl of Wash Buffer (ethanol added) into the column. Centrifuge at 6,000 xg for 30 seconds. Discard the flow-thouth and place the VB-column back in the collection tube. Centrifuge at full speed (14,000 rpm) for 3 minutes to dry the column matrix. Place dried VB column in a clean microcentrifuge tube. Apply 50 µl of RNase- free water into the center of the column matrix. Stand for 3 minutes until water is fully absorbed by the matrix. Centrifuge at full speed for 1
minute to elute purified nucleic acid. Eluted nucleic acid is free of inhibitors, nucleases, proteins and other impurities, and ready for direct application in PCR and RT-PCR or other molecular assays.

3.10.2 PCR reaction

The commercial test kit Superscript III One-step RT-PCR with Platinum Tag®, Invitrogen was used.

The system is designed for the convenience, and reproducible detection and analysis of RNA molecules by a RT-PCR. Both DNA synthesis and PCR are performed in a single tube using gene specific primers and target RNAs from either total RNA or mRNA.

This assay used for identification of avian influenza virus strain H5N1. Add the 50 µl per sample of the following components to a 0.25ml thin wall microcentrifuge tube. This is added to 50 µl of the master mix. Make a PCR reaction Master Mix as follows: (Run blank for each primer pair); 2 µl Nuclease-free Water. 25 µl 2X React Buffer mix. 10 µl 50 mM MgSO. 1 µl 10 pM Primer AIVFmix (MF, forward primer: 5'- TGA TCT TCT TGA AAA TTT GCA 3’ 276 bp. H5F: 5’ GAC TCA AAT GTA AAG AAC CTT TA 3’ 189 bp. N1F: 5’ GTT TGA GTC TGT TGC TTG GTC 3’ 131 bp). 1 µl 10 pM Primer AIVR mix (MR, reverse primer 5’ TGT TGA CAA AAT GAC CAT CG 3’ 276 bp. H5R : 5’ CCA CTT ATT TCC TCT CTG TTT AG 3’ 189 bp. N1R : 5’ TGA TAG TGT CTG TTA TTA TGC C 3’ 131 bp) were synthesized by (Yong, 2004). 10 µl RNA Template 200-800 ng. 1 µl Enzyme Mix. Mix the components, cap the tube and centrifuge briefly to the bottom of the tube. Place tube in thermocycler. was performed with a DNA Engine (PTC 200). Peltier Thermal Cycler (BIO-RAD, Hercules, lif.) as follow: a. 50ºC for 30min. 94ºC for 2min. 94ºC for 15 sec (denaturation). 55ºC for 30 sec. (annealing). 68ºC for 1 min (extension), and repeat from step 2 for 35 cycles. After 35 cycles, the DNA was given a final extension step at 68ºC for 5 minutes and 25ºC until usage.
3.10.3 Gel preparation

For gel preparation, dilute the Super Agarose 10% suspension to a required agarose concentration with an electrophoretic buffer. To prepare 50 ml of 2% gel, pour 45 ml of an electrophoresis buffer into a glass flask, and pipet 5 ml of the suspension into the buffer. Use repeated pipetting of the electrophoretic buffer to wash the residual agarose from the pipette. Mix the diluted suspension and heat it to boiling in a microwave or on a hot plate with stirring, until all the agarose is melted and dissolved to form a clear homogenous solution. Cool the solution to 60 – 70° C. and add dye if desired. Pour the molten super agarose into an electrophoretic tray, position the comb in the molten gel and allow it to harden by storing at room temperature for 1 - 1.5 hr.

3.10.4 Agarose Gel Electrophoresis of the PCR products

Remove tape from gel frame and place the gel into the electrophoresis chamber; cover the gel with 1x TBE. Label the 0.5 ml microcentrifuge tubes separately. Remove 5 µl of the PCR product from each reaction tube to a corresponding 0.5 ml microcentrifuge tube (remove PCR product from underneath oil); mix with 3 µl gel loading Dye buffer. Load 5 µl molecular weight marker (Gene ruler 100 bp DNA ladder plus) to the first well of the 2% agarose gel. Pipette 7 µl of PCR reaction, positive control and negative control to wells of the gel separately. Close lid on chamber and attach the electrodes. Run the gel and carry out electrophoresis at 300V approximately 35 minutes until the bromophenol blue marker dye has migrated 2/3 of the way down the gel.
The gels were stained in a 2 mg/L ethidium bromide staining bath for 5 -10 minutes and visualized by presence of marker and PCR product bands, and visualized by using a UV transilluminator. (Alpha Innotech, Alphalmager® HP.). It is desirable to have an ultraviolet light source emitting light at a 302 nm wavelength. Document gel with a photograph. Compare the size of the PCR-fragments with the marker.

Interpretation of results
In experiment A the expected sizes of the PCR-products for H5 is 189 bp, and N1 is 131 bp.

**Figure 6:** The result of suspension from cloacal swabs at concentration 4HAU10⁻³

Figure 6: The size of PCR products of multiplex AIV is 276 bp, 189 bp and 131bp, corresponding to the influenza A virus Matrix gene, and N1 gene, respectively. Lane M, 100 bp standard maker; lane 2,3 are positive samples; lane 1,4,5,6,7,8,9,10 are negative result. Lane N is negative control and lane P is positive control.
**Figure 7:** The result of suspension from lung organs at concentration $4HAU10^{-3}$

**Figure 7:** The size of PCR products of multiplex AIV is 276 bp, 189 bp and 131bp, corresponding to the influenza A virus Matrix gene, and N1 gene, respectively. Lane M, 100 bp standard maker; lane1,2,3,,4, 5,6,7,8,9 and 10 are negative result. Lane N is negative control and lane P is positive control.
3.11 Data management and statistical analysis

All the data were managed and analysed using STATA version 9. Survival analysis test was to be performed to compare viral isolation and identification between three diagnostic methods.

Log-rank test for equality of survivor functions is used to compare the difference between samples obtained from 2 different types of samples; between one by one of pair of methods; among 3 different methods (virus isolation and identification by using embryonated eggs, MDCK cells and RT-PCR). and a $P$ value of $\leq 0.05$ was considered significant.
4. RESULTS

The study was conducted to compare analytical sensitivity of three diagnostic methods for avian influenza virus isolation and detection of avian influenza virus of subtype H5N1 with different methods and samples.

4.1 The sensitivity among three diagnostic methods

4.1.1 The result of virus isolation by using embryonated egg. For the cloacal swabs sample, at the concentration $10^0-10^{-3}$ all samples were positive result. At the concentration $10^{-9}$, all samples were negative. For lung organs sample the concentration $10^0-10^{-3}$ all were positive samples. At the concentration $10^{-8}$ all samples were negative. (Table.2)

4.1.2 The result of virus isolation by using MDCK cells. For the cloacal swabs sample at the concentration $10^0-10^{-3}$ all samples were positive result. At the concentration $10^{-8}$ all samples were negative. For lung organs sample at the concentration $10^0-10^{-3}$ were positive all samples. At the concentration $10^{-8}$ all samples were negative. (Table.3)

4.1.3 The result of virus isolation by using RT-PCR. For the cloacal swabs sample at the concentration $10^0-10^{-1}$ all samples were positive result. At the concentration $10^{-4}$ all samples were negative. For lung organs sample the concentration $10^0-10^{-1}$ were positive all samples. At the concentration $10^{-3}$ all samples were negative. (Table.4)
Table 2. The result of virus isolation by using embryonated eggs

<table>
<thead>
<tr>
<th>Method</th>
<th>Concentration Of virus</th>
<th>Cloacal swabs replication</th>
<th>Lung organs replication</th>
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- = negative result

+ = positive result
### Table 3. The result of virus isolation by using MDCK cells

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<th>Concentration Of virus</th>
<th>Cloacal swabs replication</th>
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- = negative result

+ = positive result
Table 4. The result of virus isolation by using RT-PCR

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- = negative result

+ = positive result
4.2 Minimum detectable virus concentration

Using Kaplan-Meier survival estimates for

4.2.1 Minimum detectable virus concentration between diagnostic method (Table 5)

4.2.1.1 Minimum detectable virus concentration by using embyonated eggs was $10^{-7}$ (HA).

4.2.1.2 Minimum detectable virus concentration by using MDCK cell was $10^{-6}$ (HA).

4.2.1.3 Minimum detectable virus concentration by using RT-PCR was $10^{-2}$ (HA).

4.2.2 Minimum detectable virus concentration of sample type (Table 6)

4.2.2.1 Minimum detectable virus concentration using cloacal swab was $10^{-5}$ (HA)

4.2.2.2 Minimum detectable virus concentration using lung organs was $10^{-4}$ (HA)
Table 5. Detected proportions of samples in ten-fold concentrations in each method

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Conc.$10^{-x}$ = virus concentration in each dilution.
Table 6. Detected proportions of samples in ten-fold concentrations in each sample type

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<th>Samples</th>
<th>Conc. $10^{-x}$</th>
<th>No. of positive samples</th>
<th>No. not positive</th>
<th>Proportion detected</th>
<th>Standard Error</th>
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Conc.$10^{-x}$ = virus concentration in each dilution.
**Figure 8:** Comparison of the concentration between three diagnostic methods; using Kaplan-Meier survival estimates

The result from the graph; for the virus isolation and identification by using RT-PCR (green line) the result showed that markedly decrease of the detectable level of virus concentration which were detected at $10^3$. The result of the virus isolation and identification by using embryonated eggs (blue line) showed that the detectable level of virus concentration at $10^8$ and this result has a slightly difference from the virus isolation and identification by using MDCK cells at $10^9$. at the last dilution (red lin
Figure 9: Comparison of the concentration between two types of samples; using Kaplan-Meier survival estimates

The virus isolation and identification from cloacal swabs (blue line) and from lung organs (red line) showed that the detectable level of virus concentration were detected at $10^{-9}$ and $10^{-7}$ at the last dilution respectively (Figure 9)
4.3 Comparison of detectability level between methods

4.3.1 Comparison among difference methods

4.3.1.1 Virus isolation by using embryonated egg and MDCK cell: the detectability level between embryonated eggs and MDCK cell was statistically not significant difference. (p=0.594)

4.3.1.2 Virus isolation by using embryonated egg and RT-PCR: the detectability level between embryonated egg and RT-PCR was statistically significant difference (p=0.001)

4.3.1.3 Virus isolation by using MDCK cell and RT-PCR: the detectability level between MDCK cell and RT-PCR was statistically significant difference. (p=0.001)

4.3.1.4 Virus isolation by using embryonated eggs, MDCK cell and RT-PCR: the detectability level among 3 methods was statistically significant difference. (p=0.001)

4.3.2 Comparison between types of sample (cloacal swab and lung organs)

The detectability level between 2 types of sample (cloacal swabs and lung organs) was statistically not significant difference. (p=0.219)
5. DISCUSSION AND CONCLUSION

5.1 Discussion

In this study, three diagnostic methods compared respectively, embryonated eggs, MDCK cells and RT-PCR to find out minimum detectable avian influenza virus concentration. The selected test were similar with WHO recommend test method (WHO, 2002) only available reference describe virus isolation and identification that is recommend the test method out of these three methods. But carrying out this study found out. The lowest of minimum detectable concentration detected by using embryonated eggs and MDCK cells (HA×10⁻⁷, 10⁻⁶). Furthermore, the highest of minimum detectable concentration was received by using RT-PCR (HA×10⁻²). The samples use to work from cloacal swab and lung organs from apparently healthy chicken. WHO recommend selection of the sample (WHO, 2002). The result of minimum detectable avian influenza virus concentration by using two types of sample is cloacal swabs and lung organs (HA×10⁻⁵, 10⁻⁴).

The high sensitivity of virus isolation by using embryonated eggs (Table2.) similarly of the result with MDCK cell method (Table3.) which similar result of sample from cloacal swabs and lung organs. In addition, compared with RT-PCR test the result show the lowest of sensitivity at the highest of virus concentration. (Table 4.). Comparison of the detectability level between methods; compared virus isolation and identification using embryonated eggs and MDCK cells was not difference the result (p=0.594) between this method. Embryonated egg compared with RT-PCR method and MDCK cells compared with RT-PCR method there were difference of the result (p=0.001, 0.001) respectively. Among embryonated egg, MDCK cells and RT-PCR their were difference of the result (p=0.001). For cloacal swab sample compared with lung organs sample was not difference the result (p=0.219).
Specimens are inoculated into allantoic cavity of 9-11 day embryonated chicken eggs. High yields of virus can be harvested after 3 days of incubation and use for the routine diagnosis of influenza infection (WHO 2005d). For conventional culture: various cell lines are utilized to isolation influenza viruses. Some author recommend the use of trypsin to aid virus entry into the cell line (WHO 2005d). Virus isolation by using MDCK cells cultures is a conventional culture (Petric Martin, et al., 2006)

Compared to other reported assays (OIE and National Reference Laboratory for Newcastle Disease and Avian Influenza, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy.). Virus isolation in specific-pathogen-free (SPF) embryonated eggs or cell cultures is traditionally considered the method of choice for the detection and identification of avian influenza (AI) viruses. However, its value is limited because it is time-consuming and not cost-effective. AI is a highly contagious disease, able to spread in a susceptible population in a short period of time. Therefore, the prompt identification of an infected flock is crucial for control and eradication purposes.

Comparison among three diagnostic methods. For the results of virus isolation and identification by using embryonated eggs and Mardin Darby Canine Kidney cells (MDCK) culture received better results than RT-PCR in the present investigation. Result of the present study showed the negative result for the first passage and the positive result for the second passage in case of cloacal swabs and lung samples following the methods of embryonated eggs and MDCK cell cultures. Conversely following the RT-PCR method received negative results it may due to the virus detection from the suspension. When compared these three methods RT-PCR was time saving method than other 2 methods MDCK and embryonated eggs. As well WHO has recommended RT-PCR is the most sensitive method (WHO, 2005) But leaving the rapidity and sensitivity of the RT-PCR these 2 method of MDCK and embryonated eggs were able to detect virus at the lowest concentration (HA10⁻⁵ to 10⁻⁸) but the RT-PCR was unable to detect virus at the same lowest concentration (HA10⁻⁵ to 10⁻⁸). On the other hand, the RT-PCR did not perform well for this method in this study. Three factors may have influenced the results of this assay. First, preparation
and inoculation of antigen were not optimized by the number of samples that were inconclusive due to loss of antigens during processing. Second, store the antigen and suspension of experimental study were not appropriate for this procedure. The last factor the total amount of virus particle were not enough due to influenced the result. Additional work is needed to optimize this test method, which could be rapid method for diagnosis of avian influenza virus infection. On the other hand, molecular diagnostic techniques play a more and more prominent role in laboratory diagnosis of influenza. Direct rapid tests have also become an important tool for investigating influenza disease. In addition, suspected case from HI test should apply RT-PCR for further viral identification. The result from combined test is more efficiency.

Finally, in this study, the virus isolation and identification using embryonated eggs and MDCK cells culture should be select the appropriate laboratories, which require most infrastructure and can be performed by staff with limited knowledge of virology. Furthermore, viral culture however remains important especially for reference laboratories since it is cheap, sensitive and enables characterisation of viruses. Furthermore unlike molecular testing it is "unbiased" and can detect the unexpected new strain.
5.2 Conclusion

Methods used for influenza virus isolation and identification in birds should be specific enough to allow detection of antigenically and genetically different influenza subtypes. For the virus isolation and identification, embryonated eggs, MDCK cells and RT-PCR are widely used to detect influenza viruses directly in specimens collected from animal species susceptible to influenza virus infection. Among them, the RT-PCR is the highest virus detectable concentration. For the virus isolation and identification using embryonated eggs and MDCK cells yield similar minimum detectable virus concentration, which both of the method can detect at the lowest concentration of virus. However, there was a statistically significant difference among 3 different methods but there was not statistically significant difference between two types of samples of the cloacal swabs and the lung organs. It was not different between two sample types of virus isolation and identification.

This assay would be highly useful as a diagnostic tool to help identify of H5 Avian Influenza A virus isolate from poultry specimens, and control influenza epidemics. We can thus conclude that virological diagnosis for influenza has value for the individual laboratory, epidemiological investigations and infection control. The appropriate selection of a particular test is determined by the test characteristics and the specific diagnostic need.
REFERENCE


Hafez, H.M. (2005): Governmental regulations and concept behind eradication and control of some important poultry disease. World’s Poultry Science Journal. 61, 569-591


http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5408a1.htm
http://www.fda.gov/cdrh/oivd/tips/rapidflu.html
APPENDIXES

Appendix A: Equipment, Materials, Media and Reagents

1. Lab equipment and materials

Usual laboratory equipment and in addition: Routine servicing of the cabinet should be carried out to ensure sterile conditions inside the cabinet. Thoroughly clean the room or cabinet with disinfectant. Irradiation with UV light prior to harvesting vaccine will help kill contaminating organisms. Technicians should wear clean laboratory coats, clean hair covers, facemasks and scrub their hands with an antimicrobial soap. Sufficient work area, level table with ample surface in room that is clean, well light and well ventilated, and reasonably free of dust and drafts.

- Glassware; Pipettes; 1 ml, 5 ml 10 ml Glass Pasteur pipettes.
- Conical flasks; 50 ml 100 ml 500 ml 1 L, 2 L, Beakers; a range of sizes.
- Tubes for serial dilutions; to 10 ml. sterile 250, 500, 1000 ml. Duran bottle.
- Measuring cylinders; a range of sizes.
- Glass bottles with screw caps 20–30 ml 5–10 ml
- Assorted sterile pipettes and pipetting device
- Autoclavable containers for discarding cultures
- Class II biological safety cabinet
- Water baths, 37°C and 56°C
- Incubator, 35-37°C
- pH meter
- Vortex mixer
- Incubator, 37°C, 5% CO2
- Inverted microscope or standard microscope for the observation of cells
- Freezer, -70ºC (for long term virus storage) or -20ºC 4ºC refrigerator
- Low speed, bench top centrifuge preferably with refrigeration
- Liquid nitrogen for cell storage
- T-75 and T-25 tissue culture flasks, canted neck corning
- DNA Engine, PTC-220 DNA Engine Tetrad Cycler
- UV Transilluminature (Alpha innotech)
- Consumables; Syringes ;1 ml, 2.5 ml, 5 ml, 10 ml, Needles; 23G 23 mm, 25 G 16 mm. Tips for micropipettor. Eppendorf tubes. Cryotubes. 96-well V-bottomed microtitre plates. Plastic centrifuge tubes; 10 ml 50 ml
- Single channel and Multichannel pipettors, pipett boys, pipettes and pipettor
- Egg candling lamp. Egg incubator. Shell punch
- Bunsen burner.
- Electronic balance
- Magnetic stirrer
- Centrifuge
- Test tube racks

2. Equipment and Material for sample collection
- Scissors, forceps, plastic bottles, conical tubes, Sterile cotton wool swabs
- Maker pens, Alcohol, Cotton, lighter, Gloves
- Mask, Cap. lab coat. Ice box with ice

3. Media, reagents and chemicals
- Dulbecco’s Modified Eagle Medium (D-MEM)* GIBCO BRL, Hyclone
- Penicillin-Streptomycin, stock solution (10,000 U/ml penicillin G; 10,000 μg/ml streptomycin sulfate). Gentamicin reagent solution (50mg gentamicin sulfate/ml). Fetal bovine serum, 40 nm filtered GIBCO BRL, Hyclone. Serum Free Media, GIBCO BRL.
APPENDIX B: Stock solution

1. Buffer solution

Phosphate buffered saline (PBS)

Recipe to prepare five litres of PBS

Reagents

• Sodium chloride NaCl 40.0g
• Potassium chloride KCl 1.0g
• Potassium dihydrogen phosphate anhydrous KH2PO4 1.0g
• Disodium hydrogen phosphate anhydrous Na2HPO4 4.6g
• Distilled water to make up to 5L

Method

1. Weigh out the reagents and place in a 5 L conical flask.
2. Add distilled water to make 5 L. Mix well.
3. Check pH. Adjust to pH 7.2 to 7.4.
4. Pour into storage bottles.
5. Autoclave at 121°C for 15 minutes. Use a slow exhaust.
6. Allow to cool, then tighten the lids and label the bottles.
7. Store opened PBS, pH 7.2 at 4°C for no longer than 3 weeks.

2. Bacterial culture media

Tryptic Soy Broth (TSB)

This is a general purpose broth medium prepared for the cultivation of fastidious and non fastidious organisms.

Materials for preparing 1L of TSB

• 30 g dehydrated TSB medium
• 1 L distilled or deionized water
• Sterile 20 mL glass bottles with lids.

Method

1. Dissolve media in 1 L of water.
2. Warm slightly to dissolve completely.
3. Dispense 9 ml aliquots into the glass bottles.
4. Sterilize in the autoclave at 121°C for 15 minutes.
**Sabouraud dextrose agar**

Sabouraud dextrose agar is recommended for cultivating fungi. It is readily available and is supplied by Oxoid and Difco with instructions for its preparation. Choramphenicol is a bacterial inhibitor that can be added.

**Materials**
- Sabouraud dextrose agar 65 g
- Oxoid agar No. 1 or Difco Bacto agar 5 g
- Chloramphenicol (optional) 0.05 g dissolved in 10 ml of 95 percent ethanol.
- 1 L distilled water
- Sterile Petri dishes

**Method**
1. Suspend the agar in the distilled water. Heat to boiling point.
2. Add the chloramphenicol mixture to the agar. Stir thoroughly.
3. Dispense into 100 ml bottles.
4. Sterilize by autoclaving at 115°C for 10 minutes. Allow to cool to 50°C.
5. Pour into sterile Petri dishes. Allow approximately 25 ml for a 90 mm Petri dish.
6. Incubate plates at 37°C overnight and check for growth of bacterial contaminants.
7. Store at 4°C.

**3. Antibiotic Solution**
The recipe given is for a solution of Penicillin, Streptomycin and Gentamycin dissolved in PBS. In this manual, this solution is given the abbreviation PSG. Benzyl penicillin (Penicillin G) is a broad range antibiotic active against Gram positive and Gram negative aerobic cocci and most spirochaetes. It comes in various forms, which vary in solubility. Streptomycin is an antibiotic affective against Gram negative bacteria. Gentamycin is a broad spectrum antibiotic mainly affecting Gram negative aerobes.

**Reagents**
- Benzyl penicillin 6 g
- Streptomycin 500 mg
- Gentamycin 250 mg
- Sterile PBS 1 L

Method
1. Dissolve reagents in approximately 800 ml of PBS.
2. Make up to 1 L with PBS.
3. Cold sterilize by passing solution through a 0.2 micron filter.

Dispense into 100 ml sterile glass bottles, lid and label.

4. Anticoagulants

**Acid Citrate Dextrose (ACD)**

Reagents
- Citric Acid C(OH)(COOH)(CH₂.COOH)₂.H₂O 4.0g
- Sodium Citrate Na₃C₆H₅O₇.2H₂O 11.3g
- D-Glucose C₆H₁₂O₆ 11.0g

Method
1. Weigh out reagents into a conical flask.
2. Dissolve in 300 mL of distilled water.
3. Make up to 500 mL with distilled water.
4. Dispense into 100 mL bottles and put on lids. Do not tighten.
5. Sterilize by autoclaving at 116°C for 10 minutes. Use a slow exhaust
6. Allow to cool, then tighten the lids and label the bottles.
7. Store in the refrigerator.

**Alsever's Solution**

Reagents
- Citric acid C(OH)(COOH)(CH₂.COOH)₂.H₂O 0.055g
- Sodium Citrate Na₃C₆H₅O₇.2H₂O 0.8g
- D-Glucose C₆H₁₂O₆ 2.05g
- Sodium chloride NaCl 0.42g
- Distilled water to make up to 100 mL

Method
1. Weigh out reagents into a conical flask.
2. Dissolve of distilled water and make up to 100 mL.
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116°C for 10 minutes. Use slow exhaust.
5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator.

5. **Preparation of TPCK-trypsin stock solution**
   a. Dissolve 20 mg TPCK-trypsin* in 10 ml of dH₂O.
   b. Filter through 0.2 μm membrane.
   c. Store in aliquots at -20°C

6. **10 X TBE Buffer:**
   Tris 107.8 g
   Boric Acid 55.0 g
   EDTA(Na₂) 8.2 g

7. **Gel loading buffer**
   20% (w/v) glycerol
   1mg/ml (w/v) bromphenol blue 1Mm EDTA
DECLARATION

I, the under signed, declare that the thesis is my original work and has not been presented for a degree in any University.

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