DETECTION OF ANTIBODY AGAINST INFECTIOUS LARYNGOTRACHEITIS USING SERUM NEUTRALIZATION TEST (SNT) AND INDIRECT FLUORESCENCE ANTIBODY TECHNIQUE (IFAT) FROM SEVERAL PROVINCES IN INDONESIA

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ABSTRACT

A serological study of infectious laryngotracheitis (ILT) in layer chickens was conducted in the provinces of West Java, East Java, Bali, Lampung, and Yogyakarta. A-total of 50 sera from ILT unvaccinated chickens from 5 commercial layer chicken farms were tested. They originated from the provinces of West Java (10), East Java (10), Bali (10), Lampung (10) and Yogyakarta (10), and were tested using a serum neutralization test (SNT) and an indirect fluorescence antibody technique (IFAT). Sera with a SN titre of ^{3}1: 4 or 2 (log 2) and sera with an IFAT titre of ^{3}40 were considered positive. The results showed that the geometric mean titre (GMT) of antibodies of sera collected from West Java, East Java, Bali, Lampung, and Yogyakarta using a SN test was 4.3 (log 2) (90%), 2 (log 2) (100%), 2.1 (log 2) (100%), 4.0 (log 2) (100%) and 3.8 (log 2)(100%), respectively. However, the mean antibody titre using IFAT was 208 (80%), 32 (40%), 42 (70%), 138 (70%), and 106 (90%), respectively. This findings indicated that the SN test was more sensitive, accurate and specific for detection of antibody against ILT than IFAT. In contrary, the SN test was shown to be more time-consuming compared to IFAT.

Key words: Infectious laryngotracheitis, GMT, SN test, IFAT, layer chicken.

INTRODUCTION

Infectious laryngotracheitis (ILT) is an economically important respiratory disease of chickens caused by an alpha-herpesvirus (Hanson and Bagust, 1991). It can also affect pheasants, partridges and peafowls (Anonimous, 1996). Infectious laryngotracheitis is an acute respiratory disease of chickens characterized by coughing, sneezing, difficult breathing as major clinical signs (Tripathy, *et al,* 1980). In severe cases, birds may also show signs of gasping, dyspnea, and death (Guy, *et al.,* 1990). Infectious laryngotracheitis is considered to have a worldwide distribution and firstly reported by May and Titsler (1925) who described an outbreak in fowl in Rhode Island.

In Indonesia, the cases were firstly reported by Partadiredja *et al.,* (1982) who described field outbreaks at several commercial layer chickens in Bogor district, West Java. Since then, the disease has become widespread in several districts in West Java. Gilchrist (1992) also reported that the ILT cases occurred at village chickens in Bekasi district, West Java. Based on the distribution of the ELISA titre, Wiyono *et al.*, (1996) reported that the prevalence of ILT reactor among chickens in the districts of Cianjur, Tangerang and Karawang was 71%. The reactor sera were found in layer and village chickens, but not in broiler chickens. Furthermore, Hamid, *et al.* (2001) reported that based on pathological examination, ten out of 35 samples collected in Bogor were ILT positive, while, 11 and 5 samples out of 49 and 9 samples collected in Tangerang and Bekasi were positive respectively. Saepulloh *et al.,* (2003) showed that 14 of 95 tracheal organ samples from layer chickens located in districts of Bogor, Bekasi and Karawang were ILT positive by mean of isolation using CAMs and identified by agar gel immunodiffusion (AGID) test. Recently, Saepulloh (2003) reported that ILT occurred at a layer chicken farm in Batangas Province, the Philippines. At present there are many ILT cases in the field which attack not only layer chickens but native chickens *(ayam pelung)* kept as pet animals, admitted in a practitioner's animal clinic in Depok-West Java (Soedijar, 2001). Diagnosis in these pets was based on clinical signs and anamnesis. These findings indicated that ILT occurred at layer chickens not only in West Java but also in the Philippines.

Since 1985, twenty-five Indonesian importers and manufactures have registered ILT vaccines to the National Veterinary Drug Assay Laboratory

(NVDAL). Many mild to mildly virulent ILT strains are circulating in Indonesia. At present, several strains such as A20, A-96, Connecticut, Cover, D-805, Hudson, IVR-12, Modified Live virus, PV/09, PV/64, Samberg, and T20 have been adapted in tissue culture or in primary chicken embryonated eggs and used as ILT seed vaccine in Indonesia.

Infectious laryngotracheitis cases in Indonesia are under reported (Wibowo *et al.*, 2002). There is still a debate on the use of ILT vaccine among Indonesian farmers. A need to select an efficient procedure to detect the ILT immune status, has forced us to compare SNT and IFAT in this experiment. The first serological test developed for the detection of ILT antibodies was the agar gel precipitation test (Jordan and Chub, 1962). However, this test is now considered unreliable and relatively insensitive (Adair *et al.*, 1985). In contrary, the SNT and IFAT were shown to be time–consuming, but very reliable, accurate and suitable for quantitative measurement (Adair *et al.*, 1985 and Bauer *et al.*, 1999). The aim of this study is to detect antibody against ILT using a SNT and an IFAT,

MATERIALS AND METHODS

Sera

A-total of 50 sera obtained from 5 layer chicken farms from 5 provinces were used in this experiment. Sera were diluted 2-fold and used for SN test. Meanwhile, one layer chicken farm each, 10 sera from each farm were used in this survey. Sera for SNT were diluted 2-fold and sera for IFAT were diluted 40 times. When they gave positive results, then they were re-titrated from 80 to 640 times

Virus

The ILT virus NS-173 strain was used as standard reference virus and was obtained from the National Veterinary Assay Laboratory (NVAL), Kokubunji, Tokyo, Japan. The NS-175 strain was adapted in primary chicken embryo fibroblast (CEF) cells. A-100 $TCID_{50}$ virus was used for a SN test. While, ILT virus NS-175 strain was inoculated into monolayer coverslips and used for IFAT. When they gave more than 75% cytophatic effect (CPE), the coverslips were then harvested and fixed in cold acetone before use.

Serum Neutralization Test (SNT)

The primary CEF cells culture suspension was prepared on the flat bottom of micro-titre plates and monolayered in growth medium (Dulbeco's Minimum Eagle Medium supplemented with 0.0295 gram/ml of tryptose phosphate buffered (TPB), 0.075 gram/ml of $NaHCO_3$, 100 IU/ml of penicillin, 0.1 mg streptomycin, 0.0295 gram/ml of L-glutamin and 5% foetal calf serum). Serial two fold dilutions of 0.05 ml inactivated serum were diluted in maintenance medium (MM) (Dulbeco's Minimum Eagle Medium supplemented with 0.0295 gram/ml of TPB, 0.075 gram/ml of $NaHCO_3$, 100 iu/ml of penicillin, 0.1 mg streptomycin, 0.0295 gram/ml of L-glutamin and 1% foetal calf serum).

The same volume of virus in MM containing 100 $TCID_{50}$ ILT virus (Strain NS-175) was added to each serum dilution. Plates were incubated at 37°C for 1 hour. Then the mixed sera-virus solution was inoculated into the micro wells of the CEF monolayeres. The micro titre plates were incubated for one hour in a CO_2 incubator. MM was then added to the wells and the plates were incubated at 37°C in a CO_2 incubator for 6 days. The antibody titer was expressed as the reciprocal of the highest serum dilution, which showed complete neutralization in at least one of the wells. Titers of 4 or higher were taken to be positive.

Indirect Fluorescence Antibody Technique (IFAT)

IFAT was performed with slightly modification according to the method described by Purchase (1985). Each 40-times serum dilution was mounted onto the coverslips of monolayered cultures infected with ILT virus NS-175 strain, placed into a moisture box and kept at 37° C for 1 hour. After sensitization, the cells were washed with PBS for 15 minutes. Four units of fluorescenct isothyocianate (FITC) rabbit anti-chicken immunoglobulin G (ICN Laboratory – Israel) were added, and the cells were re-sensitized for 45 minutes. The coverslips were washed in phosphate buffer saline (PBS) for 15 minutes and glycerin buffer was mounted on the slide glass before observation under ultra violet microscope. The tested sera, which gave positive result in IFAT, were further re-titrated until 640-times according to the same procedure as mentioned above. The highest serum dilution giving fluorescent staining was recorded as the FA titres.

RESULTS AND DISCUSSION

Some researchers prefer to perform SN test using chicken-embryo liver (CELi) cells (Abbas and Andreasen, 1996; Bauer, 1999), but based on our result, ILT virus grew more easily in primary CEF cells and they showed clearer cytopathic effect (CPE), which indicated the precence of ILT virus. In addition, preparation of CEF was easier, and achieved higher yield than CELi. This finding was very useful for viral propagation especially for vaccine production.

Table 1 showed titre of IFAT for determining positive antibody against ILT was considered positive when the titers were 40-times. While less than 40-times was considered to be negative antibody, and for this value we put 20-times for them. Based on positive status, sera samples from Yogyakarta has a highest percentage (90 %) and followed by West Java (80%), Bali and Lampung (70 %) and East Java 40% (Table 1). When we put average value for IFAT, West Java occupied the highest rank (208 times) followed by Lampung (138 times), Yogyakarta (106 times), Bali (42 times) East (32 times) (Figure 1). This finding indicated that antibody statues against ILT in East Java province were negative.

SNT was more sensitive the IFAT. Table 1 showed that both the geometric mean titre (GMT) of SNT and percentage of antibody positive against ILT was higher than IFAT. Yogyakarta, Lampung, Bali and East Java were obtained 3.8 (log 2) (100%), 4.0 (log 2) (100%), 4.3 (log 2) (100%) and 4.0 (log 2) (100%), respectively, but West Java were only obtained 4.3 (log 2) (90%). Although, the percentage of antibody positive from West Java (90%) was the lowest using SNT, it still had the highest percentage compared with IFAT, 80%. The interesting ones are, that the GMT of SNT titre had delivered high titre, when the IFAT titre showed a high titer also. This finding concluded that the SN test described in these experiments was slightly greater in sensitivity than IFAT. Unfortunately, the SNT is not only time-consuming but also more expensive than IFAT. Thus, in the testing of field serum, the IFAT was found a useful confirmatory test when a rapid result was required, whilst the SN test remains the test of choice for quantification of antibody. Our result was similar to Bauer *et al*, (1999), where their result was determined positive when the titre delivered ³ 1: 4 for SNT and ³ 40 for IFAT. Unfortunately, the serum dilution did not started from 1:1 or 1:2 for SNT and 1:20 for IFAT in this study. Otherwise it can get a whole picture of ILT titre.

Table 1 : Antibodies status against ILT from 5 provinces.

Province	Serum Code	ILT-IFAT		ILT-SNT	
		Status	Titer [a)]	Status	Titer (log 2) [b)]
West Java	76	+	640	+	4
	77	-	20	+	3
	78	+	320	+	5
	79	-	20	+	2
	80	+	40	+	5
	81	+	160	+	6
	82	+	40	+	5
	83	+	640	+	7
	84	+	160	ND	ND
	85	+	40	+	2
	ab.status	80%	Mean =208	90%	GMT = $2^{4.3}$ = 19.6
East Java	86	+	40	+	2
	87	+	40	+	2
	88	+	80	+	2
	89	-	20	+	2
	90	-	20	+	2
	91	+	40	+	2
	92	-	20	+	2
	93	-	20	+	2
	94	-	20	+	2
	95	-	20	+	2
	ab.status	40%	Mean =32	100%	GMT = 2^{2} = 4.0
Bali	234	+	40	+	3
	235	+	40	+	2
	236	+	80	+	2
	237	-	20	+	2
	238	-	20	+	2
	239	+	40	+	2
	240	+	80	+	2
	241	+	40	+	2
	242	+	40	+	2
	243	-	20	+	2
	ab.status	70%	Mean = 42	100%	GMT = $2^{2.1}$ = 4.3
Lampung	670	-	20	+	ND
	671	+	160	+	5
	672	+	40	+	5
	673	+	160	+	6
	674	+	80	+	6
	676	+	640	+	7
	677	-	20	+	2
	678	+	160	+	5
	679	+	80	+	2
	680	-	20	+	2
	ab.status	70%	Mean=138	100%	GMT = $2^{4.0}$ = 16
Yogyakarta	510	+	80	+	4
	511	+	80	+	4
	512	+	160	+	4
	513	+	160	+	7
	470	+	160	+	4
	472	+	160	+	5
	505	+	160	+	4
	S7	+	40	+	2
	C4	-	20	+	2
	443	+	40	+	2
	ab.status	90%	Mean=106		GMT = $2^{3.8}$ = 13.9

Note :
ND = Not Done
a) The titre ³ 40 was considered antibody positive
b) The titre ³ 2 (log 2) was considered antibody positive

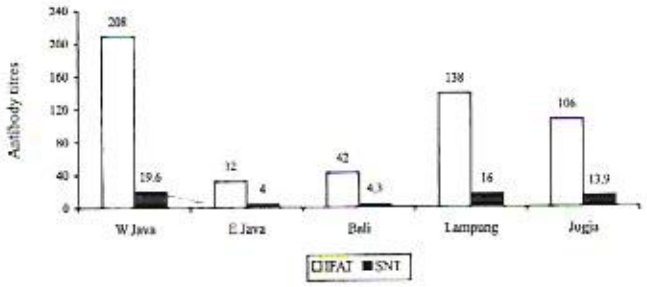

Figure 1. Average serum titre against ILT using IFAT and SNT from five Provinces in Indonesia

The poor correlation between ILT antibody titre and protection against disease is a major problem in evaluating serological test (Jordan, 1981). At the best, a synergism between neutralizing antibodies and other defense mechanisms may be assumed (Fahey *et al.*, 1983). Fahey and York (1990) considered celluler defense mechanisms (cytotoxic lymphocytes, lymphokines) as responsible for the protection of ephitelial cells in vaccinated animals. Since neither the SNT nor the IFAT results are an indication of the degree of the protection against ILT, the differentiation of sera into clearly positive and negative should generally suffice because the interest is whether a flock had contact with ILT field or vaccine virus (Andreasen *et al.*, 1990) and not the immune status of individual animals. Both tests are suitable to answer this question. For Indonesian laboratory, we may perform both tests (IFAT or SNT). Though IFAT is cheaper and time efficient it relies on the intensity of fluorescence perceived by individuals, hence interpretation of results may become more objective (Adair *et al*, 1985).

According to the above results, in the reality almost all serum samples coming from un-vaccinated chickens have antibody to ILT. This phenomenon is possible because of the occurrence of the reversion of weakened ILT virus from live vaccines given to chickens, into its virulent state. This possibility is almost similar to the occurrence of ILT outbreaks in Lipa City, Batangas Province, the Philippine in the year 2003 reported by Saepulloh (2003) where possibility of ILT outbreaks originated from chicken, which had been vaccinated with the live vaccine.

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