PREPARATION OF CELL-ASSOCIATED VACCINE FROM ATTENUATED INFECTIOUS LARYNGEOTRACHEITIS (ILT) VIRUS.

Amani Saleh, A.
Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo

ABSTRACT

The present work was carried out to develop cell-associated (CA) vaccine prepared from chicken embryo fibroblast (CEF) cells infected with the tissue-culture-modified strain of infectious laryngotracheitis (ILT). The titer of prepared vaccine was $6 \log_{10} \text{TCID}_{50}$ before and 5.7 following preservation. The vaccine was free from bacteria and other detectable viral agents by all the usual safety tests. Immunization was successful by using the prepared ILT vaccine subcutaneously in one-day-old specified pathogen-free (SPF) chicks. No clinical signs, gross and histopathological lesions related to ILT were observed in vaccinated group, as well as contact and control groups, in addition, the CA vaccine was safe when chickens were inoculated with 10 doses. The use of serum neutralization test and ELISA indicated that chicks inoculated with prepared ILT vaccine acquired high virus-neutralizing antibody titers at 14, and 21 days post-vaccination and these titers persisted in protective levels up to ten weeks post vaccination. On challenge, 93.3% protection was achieved in vaccinated groups. On the contrary all the birds in control group and chicks contact-exposed to vaccinated groups showed sever clinical signs and microscopic tracheal lesions with high mortality rates post-challenge. It is concluded that this experimental vaccine was effective for chickens, eliciting protection effect against challenge with a virulent strain of ILT virus without any clinical signs.

INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute viral respiratory disease, primarily of chickens. Economic losses attributable to ILT affect many poultry-producing areas throughout the world. Despite efforts to control the disease by vaccination, prolonged epidemics of ILT remain a threat to the poultry industry (Ivomar and Maricarmen 2007). Infectious laryngotracheitis virus (ILTV) is classified as a member of the family Herpesviridae in the subfamily Alphaherpesvirinae and continues to cause sporadic cases of respiratory disease in chickens world-wide. The virus is taxonomically identified as Gallid herpesvirus 1 (GaHV-1), genus Iltovirus, which genome is a linear double stranded DNA with 155 kb. Their exist strains of different virule-
nce and pathogenicity, but all isolates belong to one serotype (Guy and Bagust 2003).

Despite improved biosecurity and vaccination programs in recent years, ILT continues to emerge in the field on a regular basis in poultry producing areas. Evidence is mounting that most field outbreaks are caused by viruses indistinguishable from chicken-embryo-origin vaccine strains and, for that reason, broiler outbreaks in the field are often referred to be as "vaccinal laryngotracheitis" (VLT) (Dufour, 2008).

Guy and Bagust (1991) and Kotiw et al., (1995) found that the modified-live (ML) ILTV viruses, both of tissue-culture-origin (TCO) and chicken-embryo-origin (CEO), when passaged 20 times in specific-pathogen-free chickens (serial bird-to-bird passage), increased virulence was observed for CEO virus but not TCO virus. Increased mortality and increased severity and duration of respiratory disease were observed in chickens inoculated with chicken-passaged CEO viruses; only mild respiratory disease (no mortality) occurred in chickens inoculated with chicken-passaged TCO viruses. Chicken embryo fibroblast (CEF) cells has shown high levels of cytopathic effect (CPE) following ILTV infection, although the CPEs were different from those of either LMH (a chicken liver tumor line, which is used as the standard plaque assay for ILTV infection) or primary CEK cells that produce multinucleated syncytia (Byung and Jeong 2008).

Sakai, et al (1991) studied the safety and efficacy of the cell-associated (CA) vaccine prepared by chicken embryo fibroblast (CEF) cells infected with the modified-tissue-culture strain of ILTV in chickens. Over seventy percent of chickens inoculated with the CA vaccine by the subcutaneous (S.C.) or intramuscular (I.M.) route at 1 day of age were protected against challenge with a virulent strain of ILT virus without any clinical signs. Chickens vaccinated with the CA vaccine at 1 day of age acquired immunity within 6 days after vaccination, and the protection rate maintained more than 60% until 10 weeks post-vaccination. In addition, the CA vaccine was safe when chickens were inoculated with 10 doses.

On the same manner Honda, et al (1994a) observed that Chickens inoculated with the cell-associated (CA) vaccine acquired higher protective immunity to ILT. As well as, in chickens vaccinated with CA or cell-free (CF) vaccine, respectively, virus-neutralizing and IgG- and IgM-ELISA antibodies were detected in the serum, but no antibody was detected in the tracheal washes of the vaccinated chickens. More apparent antibody response was seen in chickens vaccinated with the CA vaccine than with the CF vaccine. However the cell-mediated immune responses of the chickens inoculated with the cell-associated (CA) ILT vaccine were studied by Honda, et al (1994b). Lymphocyte blastogenic response was tested by MTT assay with spleen, thymus, bursa of Fabricius and peripheral blood lymphocytes of the
vaccinated chickens. The CA vaccine induced an immunity satisfactorily in bursecto-
mized chickens indicated that the immune mechanism with CA vaccine against ILT involves mainly cell-mediated immunity. The purpose of the present study is a trial to prepare a live ILTV vaccine comprising as the effec-
tive component culture cell infected with the virus obtained by cultivation of the attenuated virus using chicken embryo fib-roblast culture cells.

MATERIAL & METHODS
1. Viruses:
1.1. Vaccinal strain:
Live attenuated tissue-culture-modified strain of ILTV was provided by Ameri-can Scientific Laboratories, Schering-Plough Corp. The virus titer was $10^6$ TCID$_{50}$/ml.
1.2. Virulent strain:
ILT Challenge Virus was supplied from the National Veterinary Services Labora-
tory, (Ames, IA). Virus was passaged 3 times in embryonated eggs and showed a titer of $10^{5.75}$ EID$_{50}$/ml

2. Cell cultures:
2.1. Chicken embryo fibroblast (CEF):
Primary chicken embryo fibroblasts (CEFs) derived from specific-pathogen-
free (SPF) chickens nine to eleven days old were prepared by methods previously des-
cribed (Lawlor and O'Brien, 1994).
2.2 Chicken kidney (CK) cell cultures:
Primary chicken kidney (CK) mon-
olayer cell cultures prepared from 1- to 6-
week-old specified pathogen-free (SPF) chickens using standard method described

by Fahey et al., (1983). CKC was used for titration of ILTV pre and post vaccine preservation.

3. Vaccine preparation:
Confluent monolayer CEFs were infected with the ILT virus using a multi-
plicity of infection of ten tissue culture infec-
tive doses (TCID$_{50}$) per cell (Meulemans et al. 1988). After the culture cells have been infected with an appropriate amount of attenuated virus, virus cultivation is kept under the ordinary conditions for four to five days at 37°C., where more than 80% of the culture cell reached a state of cytopathogenic effect (CPE), the culture cells were then trypsinized and harvested. The harvested suspension comprising floating culture cells were centrifugation (1500 rpm. 5 min.), pooled and suspended in an MEM medium containing feotal bovine serum to produce a vaccine solution with a concentration of $10^7$ cells / ml.

4. Sterility tests:
The obtained vaccine suspension was finally tested for sterility and safety by standard protocols described in OIE, (2004).

5. Preservation:
The vaccine can be preserved either in frozen or freeze-dried condition. Frozen vaccine is obtained by freezing in liquid nitrogen after suspending the culture cells with a frozen cryoprotective agent such as dimethylsulfoxide or glycerin. Freeze-dried vaccine is obtained by drying and free-
zing after suspending the culture cells with an appropriate frozen cryoprotective agent (Sakai, et al 1991). The vaccine was dilu-
ted before injection into chickens.
6. Experimental birds: Five groups (15 birds/group) one-day old specified pathogen-free (SPF) chickens were used in the experiment. The first group was vaccinated by subcutaneous injection in the neck with a final dose per bird of $10^4$ TCID50/ml (Rodri´guez, et al 2007). The second group was vaccinated with ten doses per bird for safety test of the vaccine (Sakai, et al 1991). Two groups were contact-exposed to both vaccinated groups. The last group was considered as control group.

7. Serum-neutralization test: Neutralizing antibodies to ILT-virus were determined in a micro-system using CK cell culture as described by Bauer et al (1999) and median infectious titers were recorded after 5 days, if virus controls indicated the correct virus titer of 100 TCID50. Titers were calculated according to Reed and Muench as log$_2$ values. Serum titers $\leq 1$ were considered negative.

8. Indirect immunofluorescence (IIF) assays: IIF was used as the reference method to detect the ILT-virus-infected cell culture monolayer and was performed as described by Ponntippa, et al (2002).

9. Enzyme-linked immunosorbent assay (ELISA): The blood samples from vaccinated and control groups were collected to be tested for antibody response against ILTV using ELISA test kit (BioChek B.V Holland). The ELISA was performed as per manufacturer’s recommendations. According to the description of the test kit, the relative level of antibody is determined by calculating the sample to a positive (S/P) ratio. The serum samples with S/P ratios of 0.501 or greater are considered as positive.

10. Histopathological examination: Histological examination of CEF monolayers was conducted using cover slip preparations harvested 24 to 48 hours after inoculation with virus and fixed in 10% buffered neutral formalin. They were stained with hemotoxylin and eosin and examined histologically for cytopathic effects (CPE).

11. Challenge test: Challenge with LTV was carried out to evaluate resistance of vaccinated chickens. Twenty one days post vaccination, all vaccinated and control groups were challenged with 0.1 ml of virulent LTV ($10^{4.7}$ EID$_{50}$/ml) inoculated into the infraorbital sinus. The birds were observed for a period of 10 days post-challenge and clinical scores were determined as described previously (Fuchs et al., 2005), all chick-ens were classified as healthy (0), slightly ill (1), ill (2), severely ill (3) or dead (4). Birds were euthanized, necropsied, and examined for gross and microscopic lesions (Guy and Bagust (1991). Microscopic lesions of upper and lower trachea were scored on a scale of 0-5 (normal to very severe), scores of 0 and 1 were considered negative for LTV infection; scores 2-5 were considered positive as previously described by Kirkpatrick et al., (2006).

RESULTS & DISCUSSION
Vaccination of birds with various modified forms of the ILT virus derived by cell passage and conferred acceptable protection in susceptible chickens. Because of the degree of attenuation of current ILT vaccines, care must be taken to assure that the correct level of virus is maintained; enough to provide protection, but not enough to cause disease in the flock (Byung and Jeong 2008). Following inoculation of CEF with ILTV, viral growth typically occurs after 24 to 48 hours (fig.2) and morphological changes (cytopathic effect) in cell monolayer's were observed as shown in fig.3 and fig.4 and this agreed with Portz et al., (2008) who found that ILTV isolation in fibroblast cell of chicken embryos was positive in 70% of the chicken samples and CPE was observed earlier in fibroblast cell cultures of chicken embryos (18–24 h).

The tissue culture IFA staining coincided with that of reading of the cytopathic effect (CPE) in cell monolayer's (fig.5) confirmed the presence of ILTV and this finding was in disagreement with that previously described by Schnitzlein et al., (1994) who mentioned that fibroblast cell cultures of chicken embryos were poor substrates for ILTV propagation. The virus titer of the prepared vaccine pre and post preservation was $10^6$ TCID$\text{50}$ and $10^{5.7}$ TCID$\text{50}$, respectively and the results clearly showed that no side effects of the prepared vaccine were detected either in the day old chickens received one dose or even in chickens received ten doses. There were slight respiratory reactions in some vaccin-ated chicks one week post vaccination but these reactions disappeared within 10 days post vaccination. No further clinical signs or deaths were observed in all vaccinated chicks. These results positively demonstrate the safety of the vaccine and agreed with Sakai et al., (1991). In contact-exposed groups, neither clinical signs nor morbidity were observed in both groups and this was confirmed by Rodríguez, et al (2007) who demonstrated that in spite of both ILTV vaccines CEO and TCO were capable of transmitting to contact-exposed chickens but CEO vaccine replicates and spreads faster than the TCO vaccine so the molecular epidemiologic studies suggest that the majority of outbreak strains are closely related to the CEO vaccines, whereas outbreaks with TCO-type isolates are rare.

The Virus-neutralization antibodies were detected one week post vaccination, with peak at 21 days (Table 1), one week post-challenge the rise of antibody titre was individually determined and then antibody waned to be detected at stable levels ten weeks post vaccination. Otherwise the contact-exposed groups did not induce detectable antibodies against ILTV all over the experiment. In spite of the ELISA compared to the SNT had more sensitivity and specificity, neither the SNT nor the ELISA results (Table 2) are an indication of the degree of protection against ILT due to the poor correlation between ILT anti-
body titer and protection against disease (Bauer et al. 1999), which is a major problem when evaluating the results of ILT serology.

However, the serum antibody levels against ILTV did not correlate with the protection rate, immunologic methods to detect these levels are not appropriate for evaluating vaccine efficacy. The efficacy of ILTV live vaccines can be assessed by the survivability of vaccinated chickens at 14 or 21 days after challenge with a virulent strain of ILTV (Han and Kim 2003). Honda, et al (1994a) injected the chickens with culture cells infected with ILTV and unexpectedly found that the cells constituted a very effective vaccine which can be injected subcutaneously, so to confirm the effectiveness of the prepared vaccine in this study, challenge test was carried out on the chickens which had received both one and ten doses. These chickens maintained enough immunity to be protected from the virus infection as no clinical symptoms were observed in vaccinated groups except one bird showed signs of illness (score 1) without any mortality rate (even the group which received ten vaccinal doses) up to ten weeks post vaccination.

However, no gross or microscopic lesions (score 0-1) were observed in necropsied birds, except the individual case in the vaccinated group that showed signs of illness exhibited microscopic lesions (score 2). On the contrary, post challenge test the control-group and contact-vaccinated chickens exhibited 100% morbidity and 85-95% mortality (Table 2) with signs of conjunctivitis, coughing, and lesions of mucoid, haemorrhagic tracheitis which is the characteristic symptoms of infectious laryngotracheitis with severe microscopic tracheal lesions (score 4-5) (fig.6).

Generally, insufficient effectiveness is a problem in the case of vaccination to newly hatched chicks because of the antibodies transferred from the mother. It is presumed that the culture cells, which cover the infectious laryngotracheitis virus in this vaccine, prevent adverse effect from the maternal antibodies (Honda et al., 1990 and Sakai et al., 1991). However, the present vaccine exhibits adequate immunoefficiency effect even in the presence of the maternal antibodies in chickens injected with the vaccine. Furthermore, there still remains the possibility that the vaccine applied to the eyes may have side effects, as the vaccine viruses were replicated in eye conjunctiva and trachea and spread to contact-exposed chickens (Rodríguez, et al 2007).

Additionally, chickens older than 14 days experience additional stress when caught for vaccination. Moreover, they can move about quite quickly at this age, so that much work is needed to catch them. The vaccination work thus becomes a major burden for breeders who raise a large number of chickens, so the major advantage of this present vaccine is that it exhibits high immunization effect and safety, and thus can be used to newly hatched chicks. From these results, the present vaccine was confirmed to be very effective.
when applied subcutaneously to day-old-chick in preventing infection by infectious laryngotracheitis virus.

**Table (1): Geometric mean titers of ILTV neutralizing antibodies in sera of chicken groups measured by SNT.**

<table>
<thead>
<tr>
<th>Week post vaccination</th>
<th>Geometric mean antibody titers* / Chicken groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.42</td>
</tr>
<tr>
<td>2</td>
<td>11.88</td>
</tr>
<tr>
<td>3**</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>55.7</td>
</tr>
<tr>
<td>5</td>
<td>84.4</td>
</tr>
<tr>
<td>6</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>57.1</td>
</tr>
<tr>
<td>8-10</td>
<td>45.25</td>
</tr>
</tbody>
</table>

*= The reciprocal of the end point of serum dilution which neutralized and inhibited the CPE of 100 TCID<sub>50</sub> of ILTV.

** = Challenge test
Gr. A = Chicken group received one dose of ILT vaccine.
Gr. B = Chicken group contact-exposed to group A.
Gr. C = Chicken group received ten doses of ILT vaccine.
Gr. D = Chicken group contact-exposed to group C.
Gr. E = Unvaccinated control group.

**Table (2): ELISA antibody titers to laryngotracheitis virus in vaccinated and contacts chicken groups**

<table>
<thead>
<tr>
<th>Week post vaccination</th>
<th>Geometric mean antibody titers* (log&lt;sub&gt;10&lt;/sub&gt;) / Chicken groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td>2</td>
<td>3.91</td>
</tr>
<tr>
<td>3**</td>
<td>4.04</td>
</tr>
<tr>
<td>4</td>
<td>4.76</td>
</tr>
<tr>
<td>5</td>
<td>4.38</td>
</tr>
<tr>
<td>6</td>
<td>4.1</td>
</tr>
<tr>
<td>7</td>
<td>3.89</td>
</tr>
<tr>
<td>8-10</td>
<td>3.85</td>
</tr>
</tbody>
</table>

*= Samples with log10 titer = 3.03 or greater are considered positive. ** = Challenge test
Gr. A = Chicken group received one dose of ILT vaccine. Gr. B = Chicken group contact-exposed to group A. Gr. C = Chicken group received ten doses of ILT vaccine.
Gr. D = Chicken group contact-exposed to group C. Gr. E = Unvaccinated control group.
Table (3): Protection of vaccinated and contacts groups post challenged with virulent ILTV.

<table>
<thead>
<tr>
<th>Chicken groups</th>
<th>Morbidity</th>
<th>Mortality</th>
<th>Clinical score</th>
<th>Microscopic score</th>
<th>Positive ILTV-specific antibodies</th>
<th>Protection rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. A</td>
<td>1/15</td>
<td>0/15</td>
<td>1</td>
<td>2</td>
<td>15/15</td>
<td>93.3</td>
</tr>
<tr>
<td>Gr. B</td>
<td>15/15</td>
<td>11/15</td>
<td>4</td>
<td>4</td>
<td>6/15</td>
<td>0</td>
</tr>
<tr>
<td>Gr. C</td>
<td>0/15</td>
<td>0/15</td>
<td>0</td>
<td>1</td>
<td>15/15</td>
<td>100</td>
</tr>
<tr>
<td>Gr. D</td>
<td>13/15</td>
<td>8/15</td>
<td>3-4</td>
<td>4</td>
<td>7/15</td>
<td>13.3</td>
</tr>
<tr>
<td>Gr. E</td>
<td>15/15</td>
<td>12/15</td>
<td>4</td>
<td>5</td>
<td>0/15</td>
<td>0</td>
</tr>
</tbody>
</table>

Gr. A = Chicken group received one dose of ILT vaccine.  
Gr. B = Chicken group contact-exposed to group A.  
Gr. C = Chicken group received ten doses of ILT vaccine.  
Gr. D = Chicken group contact-exposed to group C.  
Gr. E = Unvaccinated control group.

Fig. (1): Normal chicken embryo fibroblast (CEF). (H&E) X100.
Fig. (2): CPE on chicken embryo fibroblast (CEF) 48 hr post infection with ILTV. (H&E) X 200.
Fig. (3): Infected CEF (72hr P/I) showed abnormal characteristics as round, ballooned cells fused with others to form giant cells. (H&E) X200.

Fig. (4): Infected with ILTV 96 hr post infection showing syncytial cell formation. (H&E) X400.

Fig. (4): IFA reactions of ILTV infected CEF stained with fluorescein 48 hr post infection.

Fig. (5): Trachea showing desquamation of tracheal mucosa with detached cells, inflammatory cells and RBCs within the lumen (score 4). (H&E) X 200.
**Acknowledgements:** I would like to thank Dr. Ahmed, Z. El-Herrawy prof. of para-cytology. Water Pollution Research Dep. N.R.C. for his help in this research.

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تحضير لقاح نسيجي مرتبط بالخلايا من الفيروس المستضعف لمرض إلتهاب الحنجرة والقصبة الهوائية المعدي

د. أماني على صالح
معهد بحوث الامصال واللقاحات البيطرية – العباسية – القاهرة

في هذه الدراسة تم تحضير لقاح نسيجي مرتبط بالخلايا من الفيروس المستضعف لمرض إلتهاب الحنجرة والقصبة الهوائية المعدي بتمريره على خلايا أجنحة الدجاج الأولية. وكانت القوة العيارية للقاح هي 0.6، 1.7 قبل وبعد حفظ اللقاح على التوالى. وبعد إجراء اختبارات النقاوة للقاح المحضر، تم تصميم مجموعات من الكنائس (عمر يوم) الخالية من المسببات المرضية عن طريق الحقن تحت الجلد خلف الرقبة. وقد لوحظ عدم ظهور أي أعراض مرضية أو تغيرات في الأنسجة عند الفحص العينى والمجهري للقصبة الهوائية والرئة في الكنائس المحصنة بكل من الجرعة المقررة وعشرة أضعاف تلك الجرعة بالإضافة إلى الكنائس الملاصقة والضابطة مما بثت أن اللقاح أمن. وبإجراء كل من اختبار التعادل المصلي واختبار الإلزام على امصال الكنائس المحصنة، وجد أن الطيور اكتسبت رد فعل مناعى عالى ضد المرض في الأسبوع الثاني والثالث بعد التحصين والذي يستمر حتى الأسبوع العاشر. وبإجراء اختبار التحدي أظهرت الطيور المحصنة نسبة حماية تصل إلى 93.3% وعلى العكس اظهرت الطيور غير المحصنة والخالية للمجموعة المحصنة والضابطة أعراض شديدة للمرض ومعدلات نفوق عالية. وعلى ذلك يمكن اعتبار أن اللقاح المحضر فعال وأمن لتحسين الطيور ضد عدوى فيروس إلتهاب الحنجرة والقصبة الهوائية المعدي.