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Short Communication

Moderately-high humoral antibody responses to a H5N2 inactivated vaccine did not suppress shedding of highly pathogenic H5N1 Avian Influenza virus during challenge

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INTRODUCTION

Since the outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in poultry in 2000 to 2004, various countries have adopted several strategies to control or eradicate the disease. Some have chosen stringent measures such as killing and destruction of infected poultry. However, as these methods proved to be expensive and biosecurity measures and culling cannot be implemented to successfully control or eradicate the disease for some countries, an alternative method, is therefore, vaccination. Vaccination is also one of the tools recommended by international health organisations in controlling AI (OIE). For this reasons only two types of vaccines have been currently approved, (i) heterologous low pathogenic inactivated vaccines and (ii) recombinant vaccines (Swayne et al., 2000). Since the emergence of H5N1 in Asia, several heterologous inactivated vaccines have been developed and tested against H5 and H7 influenza viruses in poultry and the use of heterologous inactivated H5N2 vaccines had been reported in chickens in Hong Kong (2002 - 2006), Pakistan (2006), India (2006), Russia (2005), Egypt (2006), in ducks, geese and chickens in China (2004) and Vietnam (2005) to name a few (Swayne et al., 2001; Swayne et al., 2006; Swayne 2009). Although these vaccines can protect poultry from clinical disease, sterile immunity is not achieved under field conditions, allowing for undetected virus spread and evolution under immune cover (Fuchs et al., 2009). However, controlling highly pathogenic H5N1 using inactivated highly pathogenic H5N1 vaccines are not permissible for fear that residual viruses that are not fully inactivated can cause outbreaks. Despite this, in 2003, Indonesia, however, started using an autologous inactivated H5N1 vaccine to control the rapid spread of H5N1 in its poultry population (Swayne, 2009). However, they showed that the inactivated homologous H5N1 vaccine being completely protective than the H5N2 virus vaccines against H5N1 challenged. In using inactivated heterologous vaccines, where the virus strain used to make the vaccine is of the same H subtype as the challenging field virus the clinical protection and the reduction or viral shedding are ensured by the homologous H group (Capua and Marangon, 2003). Similar HA subtype or high percentage homology (90 - 96%) between the vaccine strain and the circulating strain are critical factors for the efficacy of the vaccine. However, other factors such as antigen quantity and content and the adjuvant used for the efficacy of the inactivated vaccines are also important (Swayne et al., 1999; Wood et al., 1985). The ability of the heterologous vaccine to provide protection against mortality and morbidity, reduce cloacal and oropharyngeal shedding and ability to prevent viral spread to other vaccinated or susceptible birds have been considered as important factors for protective efficacy of the vaccine. The aim of the study is to determine the potency and efficacy of the inactivated H5N2 vaccine developed, and the ability of the vaccine to invoke sterile immunity as depicted by shedding of challenge virus, after challenged with a highly pathogenic Malaysian strain of H5N1 virus.

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SUMMARY

The potency and efficacy of an avian influenza (AI) H5N2 inactivated vaccine that was developed at Veterinary Research Institute, Ipoh was tested. The percentage sequence identity of the HA gene of the H5N2 vaccine virus to the challenge virus [A/chicken/Malaysia/5858/04 (H5N1)] was 88.2% by nucleotide and 90% by amino acid sequences similarities, respectively. As for the HAI segment, the nucleotide sequence similarities were 88.3 % and by amino acid sequence 87.7%. For potency testing, the heterologous killed H5N2 AI vaccine, formulated as an oil emulsion was administered only once subcutaneously in twenty five two-week old commercial broiler chickens. The HI antibodies were not detectable at week 1 post vaccination. The HI GMT attained was 30, 63, 54 and 32 by week 2, 3, 4, 5, and 6 post vaccinations. Efficacy study was conducted on ten SPF chickens at week 3 post vaccination. 60% of the birds (6/10) with HI titres ≥ 64 - 128 survived the challenged. H5N1 challenge virus was reisolated from all the birds with HI titre ≤ 32 that died, and each of the birds that survived with HI titres of 64 and 128, from the oropharynx and cloaca at day 3 post challenge. This vaccine protected 60% of chickens against mortality and did not prevent shedding after challenged with a HPAI H5N1 virus.

Keywords: Avian Influenza, Virus, Vaccine

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MATERIALS AND METHODS

Viruses

The vaccine virus A/duck/Malaysia/8443/04 H5N2 was isolated from the cloacal swab of a duck in a routine surveillance study in the country. During isolation of the virus in 9 - 11 day-old SPF embryonated eggs, the HA activity was detected as early as the first passage. However, it took 4 passages before the virus kill the SPF embryonated eggs. The virus was non-pathogenic as determined by the intravenous pathogenicity index (IVPI) by the standard procedure (Council Directive 92/40/EEC (1992) Off. J. Eur. Communities L167, 1 - 16). The sequence of the HA cleavage site is TIGEC PKYV KSRLVL AKGLRN VPQ --- RETRGLF.

The challenge virus strain used was A/chicken/Malaysia/5858/04 H5N1. This virus was isolated from chickens during an outbreak in Malaysia in 2004. The virus had an intravenous pathogenicity index (IVPI) of 3.0, where 4 weeks old chickens inoculated with this virus died within 24 hr (determined by AAHL, Geelong, Australia, the OIE Reference Centre for Avian Influenza). The presence of multiple basic amino-acids at the HA cleavage site sequence of TIGEC PKYV KSRLVL AKGLRN VPQ --- RETRGLF indicated the high pathogenicity of the virus. The lethal dose of the virus was determined to be 10^3 EID_50/0.1 ml where it causes 100% mortality of SPF chickens within 48 hr post-infection. All laboratory and animal experiments using the highly pathogenic H5N1 virus was performed in a BSL-3 facility of the Veterinary Research Institute, Ipoh, Malaysia.

Sequencing of the Haemagglutinin gene

PCR was carried out to amplify the full length HA gene of the H5N1 challenge virus A/chicken/Malaysia/5858/04 H5N1 and the A/duck/Malaysia/8443/04 H5N2, using HA specific primers as previously described (Hoffmann et al., 2001). The products were cloned into TOPO PCR vector and sequenced. Sequences were assembled and edited using Staden Package, Pairwise sequence alignments and the nucleotide and amino acid sequence were compared using the Bio-Edit 7 and Genetyx-Mac programmes.

Preparation of the H5N2 Vaccine

The master seed and working viruses of the duck isolate were prepared in SPF eggs. A preliminary batch of vaccine virus was produced by inoculating a batch of 500 SPF eggs with 10^3 EID_50/0.1 ml (This dose was found to give the highest virus titre at day 3 post inoculation). Vaccine virus infected eggs were incubated for 3 days. The undiluted allantoic fluid containing virus was inactivated for 18 hr with B-propiolactone at 0.01 %/v and adjuvanted with 10%Montanide™ gel. The pre-activation infectivity titre and the HA titre of the vaccine virus were 10^7.3 EID_50/0.1 ml and 128 HAU respectively.

For determining the potency of the H5N2 inactivated vaccine, twenty five three-day old commercial broilers which were not vaccinated with any poultry vaccines were reared until they reached the age of two weeks-old. The birds were wing-banded and reared in a non-infectious animal housing unit.

Vaccination response-experiment

At two weeks old, the birds were immunized with the H5N2 vaccine. A dosage of 200µl was injected subcutaneously (SQ) per bird, and the serology of all the immunized birds were evaluated every week for a period of 6 weeks.

HI assay

The detection of antibodies after vaccination was studied by the HI assay performed according to the WHO manual on Animal Influenza diagnosis and Surveillance (WHO/CDS_CSR/NCS/2002.5). Serum samples were diluted 2 fold, with the initial serum dilution at 1:2. Titres > 3 log_2(8) are considered positive. The serological response was evaluated for all birds before and after vaccination. The HI test was performed in V-bottom 96 well microtiter plates with 8 HAU/50µl of homologous inactivatedH5N1 antigen per well.

Challenging vaccinated birds with H5N1 virus

In another experiment, ten two-week old SPF chickens (raised at SPF chicken facility of Veterinary Research Institute, Ipoh, Malaysia) were vaccinated with 200µl of the H5N2 vaccine via the SQ route. At 3 weeks post vaccination (based on 100% seroconversion from earlier potency study), the birds were challenged with 200µl containing 10^3 EID_50/bird of the H5N1 virus via the intranasal route. Challenging of the chickens with HPAl H5N1 virus, was conducted in a negative pressure isolator cabinet ventilated with HEPA-filtered air in a NATA-certified biosafety level-3 facility of Veterinary Research Institute, Ipoh. Water and feed were provided at libitum. Five SPF birds that had not been vaccinated with the H5N2 vaccine were also challenged with the same dose of virus. Clinical signs were monitored daily for one week post-challenged. Cloacal and oropharyngeal swabs of each of the chickens were sampled at 3 days post challenge for H5N1 virus re-isolation. Virus isolation was performed in 9 - 11 days old SPF embryonated eggs using standard procedures (OIE, 2012). The presence of H5N1 challenge virus was detected using the HA test and confirmed using specific H5N1 haemagglutination-inhibition (HI) serological test. Three passages were undertaken and HA test performed at each passage before the samples were considered negative.
RESULTS

HA gene sequence

Compared to the challenge virus, the percentage sequence identity of the HA gene of the vaccine H5N2 and challenge virus H5N1 was 88.2% by nucleotide sequence (Figure 1) and 90% by amino acid sequence. As for the comparison of the HAI segment, the nucleotide sequence similarities were 88.3% and by amino acid sequence was 87.7% similarities.

Vaccination response

Table 1 and Figure 1, showed the HI GMT and the percentage of birds attaining positive HI titres at various weeks after a single vaccination dose with the H5N2 vaccine at two weeks old. By week 1 post vaccination (pv), HI antibodies were not detectable in any of the 25 vaccinated birds. By week 2 pv, 60% of the birds were positive (HI ≥ 8) for HI antibodies. By week 3 pv, 100% of the birds seroconverted with positive HI titres; however, the titres were not high, where only seven birds had HI titres of 64 and 128. By week 4 pv, the percentage of birds with positive titre reduced to 96%, however, achieved the highest GMT of 200 where 18/25 birds (32%) attained high HI antibody titres of 64-512; and by week 6 pv, the antibodies waned off to a GMT of 32 with 72% of the birds having positive titre. However, the probable percentage of protection against mortality, based on a protective titre of ≥ 40 (Kumar et al., 2007), if birds were challenged with a pathogenic H5N1 strain would be 28%, 72%, 4% and 4% at week 3, 4, 5 and 6 post vaccination respectively (Figure 1).
Table 1: Relationship of the potency, HI Geometric Mean Titre (GMT) and probable percentage protection afforded by the H5N2 vaccine on 25 commercial birds. Chicks were vaccinated at two weeks old and each bird was inoculated SQ with 200µl vaccine (pre-activation titre: 10^{7.3} EID_{50}/0.1ml).

<table>
<thead>
<tr>
<th>Week post vaccination</th>
<th>No of birds</th>
<th>HI titre</th>
<th>GMT</th>
<th>a/b (Percent) positive HI titre : HI ≥ 8</th>
<th>Probable percentage of protection based on a protective titer HI value ≥ 40 (Kumar et al. 2007)</th>
</tr>
</thead>
<tbody>
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<td>0 (before vaccination)</td>
<td>25</td>
<td>&lt;2</td>
<td>0</td>
<td>0/25 (0%)</td>
<td>0%</td>
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<td>1</td>
<td>25</td>
<td>&lt;2</td>
<td>0</td>
<td>0/25 (0%)</td>
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<td>2</td>
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<td>&lt;2</td>
<td>30</td>
<td>15/25 (60%)</td>
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<td>63</td>
<td>25/25 (100%)</td>
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</table>

**Challenged response and shedding**

Only ten birds were used for the challenge and shedding studies as there was limited space in the BSL-3 cabinet for ease of handling the chickens. As was observed in the potency study, the rise of humoral HI antibodies were slow, i.e. it took three weeks post vaccination for all birds to seroconvert. Challenge was therefore done at week 3 pv, to ensure that all birds have antibody titres by then. The birds had pre challenged HI titres ranging from 8 – 128 i.e two birds with HI titre of 8, two birds with HI titre of 32; four birds with HI titre of 64 and two birds with HI titre of 128 (Figure 2). All four birds with HI titre ≤ 32 died during challenged. The birds died within 3 - 4 days post challenged. The six birds with HI titre ≥ 64 survived challenged with no clinical signs observed. Shedding was evaluated at only one time i.e. at 3 days post-challenged. Challenge H5N1 virus was excreted in the oropharynx and cloaca when examined at 3 days post challenged in 7/10 birds (70%), i.e from four birds that died at 3 - 4 days post challenged, in one bird with HI titre of 64 and one bird with HI titre of 128. Birds showed signs of depression, ruffled feathers and loss of appetite before death.

**DISCUSSION**

The Government of Malaysia does not adopt the policy of routine vaccination of poultry against avian influenza. However, in a worst case scenario, the government recognizes the potential of vaccination as a complementary measure in the control and eradication of HPAI, or at least for the vaccination of expensive or rare exotic birds. In view of this, a pilot batch of vaccine was prepared using a low pathogenic A/Duck/Malaysia/8443/04 (H5N2) virus. In our study, even at a high pre-activation titre of H5N2 virus of 10^{7.3} EID_{50}/0.1 ml, and adjuvanted with 10% montanide gel (a potent adjuvant), the HI titres invoked with a single vaccination of this vaccine is moderately low
with the highest HI titre achieved was 512 in only one bird. It was only after three weeks post vaccination that 100% seroconversion was observed.

The HI antibody response could not be detected at 1 week post vaccination, however the GMT achieved its peak of 200 by week 4 pv but the antibodies waned off quickly by week 6 pv. This low-moderately low potency of the vaccine had also been shown by Kumar et al. (2007), in chickens vaccinated with a reverse genetic H5N3 isolate where the HA gene was derived from A/chicken/Vietnam H5N1. The chickens achieved suboptimal antibody response of HI < 40. He also showed that chickens with serologic responses of > 40 were protected against challenge with the H5N1 virus. He also showed that, at this protective titre, the virus could still be reisolated from one out of the 62 birds tested. In our potency study, using Kumar's value of HI > 40 as the protective titre, at week 3 and 4 post challenged, the probable protection afforded would only be 28% and 72% respectively. However, in our challenged study, using ten SPF chickens, 60% protection was afforded when chickens were challenged at week 3 post vaccination. We were also able to reisolate the challenge H5N1 virus in 7/10 birds. In conclusion, the H5N2 inactivated vaccine invoked only sub-optimal humoral HI antibody titres, not enough to protect at least 80% of the birds against challenge, although the HA protein share 90% amino acid homology with the challenge H5N1 virus. According to Swayne et al., 1999, the degree of protection of inactivated vaccines is not strictly correlated to the degree of homology between the HA gene or protein of the vaccine and challenge strains, therefore the vaccine can still be improved to achieve a higher degree of clinical protection and a better reduction of shedding i.e. by increasing the antigen mass of the vaccine. Due to space constrains of the BSL-3 facility, this is only a preliminary and small study, and therefore there were insufficient numbers of birds at all the various HI titres to make statistical inferences of protection associated with titres.

REFERENCES


INTRODUCTION

Escherichia coli, also known as E. coli, is an ubiquitous organism and one of the leading causes of diarrhoea in suckling piglets, especially in piglets reared under intensive management system (Fürer et al., 1982). Neonatal diarrhoea associated with E. coli is most commonly observed in piglets aged from 0 – 4 days (Loh et al., 2006; Schwartz, 2009). The severity of neonatal diarrhoea associated with E. coli is also age-related, and the highest incidence of life-threatening diarrhoea occurs during the first 2 to 5 days of life, with less serious diarrhoea occurring later (Loh et al., 2006; Schwartz, 2009). Neonatal diarrhoea and deaths caused by enterotoxigenic Escherichia coli (ETEC) were observed in many herds, especially in piglets farrowed by gilts, whereas piglets from older sows showed lower vulnerability (Too, 1997; Riising et al., 2005).

The high pre-weaning mortality rate in Malaysia of about 12% of total piglets born alive has not changed over a period of 15 years (1981–1996) and this post a major problem in the swine industry (Loh et al., 1999). Heavy losses have been reported in piglets during the first week of life in Malaysia and many were thought to be as a result of E. coli infection, or commonly known as colibacillosis, where more than 95% of E. coli isolated from diarrhoeic piglets had developed multiple antibiotic resistances, and more than 50% showed resistance to 10 types of antimicrobials tested (Loh et al. 2006) can impose a high economic impact to the producers.

Therefore, it is highly recommended to prevent this disease than to continuously fight it (Holden et al., 2006).

Antibodies in colostrum provide passive protection to suckling piglets from sows that have built up immunity to specific E. coli strains (Carr, 2006). Active immunization of sows effectively provided protection to the newborn piglets through the transfer of antibodies via colostrum (Riising et al., 2005). The protection of newborn pigs against E. coli infection by sow vaccination has been a well-established practice in Denmark for more than 2 decades (Riising et al., 2005). This practice is not commonly done in farm conditions in Malaysia.

This study has its aim to observe the effects on the neonatal (first week) and pre-weaning (second and third week) performance of piglets sowed by gilts vaccinated with E. coli vaccine in a farm in Perak, Malaysia for the following parameters: (1) Diarrhoea percentages, (2) Growth Performance–average body weight and average daily gain and (3) Mortality rate.

MATERIALS AND METHODS

Study animals

The field trial was carried out as a prospective study involving the progeny of 35 in-house bred Landrace-Yorkshire gilts artificially inseminated with semen from in-house bred Duroc boars, randomly assigned to Treatment Group – 16 gilts (vaccinated with E. coli bacterin – Treatment Group) and Control Group – 19 gilts, from a farm in Perak with a sow population of 4,000. Gilts were
placed into standard size farrowing pens in an intensive open house system and each litter of respective gilts consisting of siblings were monitored for 21 days. Cross fostering of piglets to other sows was strictly prohibited in this study. Good farm husbandry management and facilities (heating lamps) were equally provided to both groups.

**E. coli vaccine**

The vaccine tested is an inactivated vaccine (Neocolipor–Merial) containing recombinant porcine *E. coli* strains with F4 (F4ab, F4ac, F4ad), F5 adhesins and inactivated field porcine strain with F6, F41 adhesins. This covers the main adhesins that dominate in neonatal diarrhoea (Runnels et al., 1987).

**E. coli vaccination**

Vaccination of the treatment group was done prior to the study period. The first dose was given by intramuscular injection in the neck region behind the ear, 5 – 7 weeks before farrowing, with the booster dose given 2 weeks before farrowing. The newborn piglets were allowed to suckle colostrum.

**Evaluated Parameters**

The numbers of diarrhoeic piglets observed in each litter were recorded daily for 14 days. This was done by the visual observation of every piglet’s anus for stains of diarrhoea. Litter size and the cumulative weight of siblings were recorded on Days 1, 7, 14 and 21 using an analogue weighing scale, with an accuracy of 0.5 kg. The average body weight and the average daily gain were computed. Piglets that died during the study were recorded as mortality throughout the 21 days of study. Although, the cause of death was not thoroughly investigated, piglets crushed by sow or died due to external trauma were excluded from the study. All data collected was analysed using Mann-Whitney-U Test at 95% confidence level.

**RESULTS AND DISCUSSION**

**Diarrhoea percentages**

The results for the average percentage of diarrhoea episodes per litter for Week 1 and 2 are shown in Figure 1. There were no statistical differences between groups (p > 0.05), despite Treatment group exhibiting lower diarrhoea episodes in Week 1 and a non-significant slightly higher diarrhoea score in Week 2 compared to the Control group. These findings can be attributed partially to the higher mortality of piglets in the Control group in Week 1 (Figure 1). Piglets with *E. coli* infection will most commonly suffer from severe neonatal secretory diarrhoea, leading to the death. Piglets that had died from the study were omitted, as no further data could be obtained from them after such point. These piglets may likely be the ones greatly affecting the diarrhoea scores, and subsequently the body weight parameters in the study.

The average diarrhoea percentage per litter was further analysed on a daily basis for the first week (Figure 2). At day 1, control group piglets exhibited significantly more diarrhoea (p < 0.05) and had a higher peak percentage when compared to the piglets in the treatment group (i.e. 19.0% versus 13.8%, respectively). On day 2, peak diarrhoea episodes in both the treatment and control group piglets were observed.

![Figure 1: Average percentage of diarrhoea episodes per litter in Treatment and Control groups](image1)

![Figure 2: Average daily percentage of diarrhoea episode per litter during week 1 in Treatment and Control groups](image2)
The average percentages of diarrhoea per litter for the subsequent days were not significantly different (p > 0.05) and gradually declined in frequency in both groups. The peak in diarrhoea score at day-2 with subsequent declining pattern coincides with the manifestation of E. coli infection, when peak diarrhoea manifestation is usually between 2 to 5 days of age (Schwartz, 2009). Other diseases causing neonatal diarrhoea were less likely, judging from the clinical signs and diarrhoeic patterns (Schwartz, 2009).

**Growth Performance**

The average body weight of the piglets from the Treatment and Control groups are shown in Figure 3. There were no significant differences in the average body weights of piglets (p > 0.05) in both Treatment and Control groups at all the ages monitored, i.e. day-1, day-7, day-14 and day-21, despite the higher body weights of 5.7% in the Treatment group over the Control group, i.e. 5.01kg and 4.74kg respectively at Day 21.

![Figure 3: Average body weight per piglet in Treatment and Control groups](image)

The average daily gain (ADG) of the piglets from gilts vaccinated with E. coli bacterin vaccine and the control group are shown in Figure 4. Similar to the average body weight, the ADG of piglets in the Treatment group were higher than in the Control group, however the differences were not significant (p > 0.05). Piglets in the Treatment group had an overall better daily gain of 5.9% over piglets in the Control group from day 1 – 21.

![Figure 4: Average Daily Gain of the piglets in Treatment and Control groups](image)

### Mortality Rate

The mortality rate observed for Treatment and Control groups at Weeks 1, 2 and 3 are shown in Table 1. Piglets in the Treatment group had a significantly lower mortality (p < 0.05) during the first week as compared with the Control group (2.6% and 5.9%, respectively). It may be inferred that the piglets who were highly contributing to the diarrhoea score in Week 1 in the Control group did not survive to the second week, whereas, in the Treatment group, piglets having diarrhoea in Week 1 surviving into the second week were recorded for the incidence of diarrhoea. The surviving piglets from a diarrhoeic episode may perform slightly slower than normal healthy piglets (Edfors-Lilja et al., 2000), subsequently affecting both the diarrhoea score and the growth parameters. There was no difference in piglet mortality in both groups during the second and third week of age. However, the mortality difference was significantly lower (p < 0.05) in the Treatment group as compared with the Control group for the overall period of 1 to 21 days (i.e. 3.9% and 8.6%, respectively). Previous field trials carried out in Austria and Norway for the same E. coli vaccine showed piglet mortalities of 4% for the Treatment groups and 10% in the Control group, similar to the results in this study. This may suggest the reproducibility of the results under farm environments.

Piglets in the Control group had consistently higher neonatal mortality rates when compared to the treatment group with a peak at days 3 and 4 (1.6%) at first week of age. The treatment group had peak mortalities at day 4 with a neonatal mortality rate of 1.3% (Table 2).

### Table 1: The effects of E. coli vaccination on mortality rate in Treatment and Control groups

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1.30</td>
<td>1.60</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1.07</td>
</tr>
<tr>
<td>Total Mortality Rate</td>
<td>3.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.56&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d</sup> Values with different superscripts are significantly different (P <0.05)
Table 2: Daily mortality rate of Treatment and Control group from Day 1 to Day 7

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment (%)</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.53</td>
</tr>
<tr>
<td>2</td>
<td>0.65</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>1.61</td>
</tr>
<tr>
<td>4</td>
<td>1.30</td>
<td>1.61</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.53</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0.53</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

This study did not control for factors that include: (a) the unavailable data from diarrhoeic piglets that died in the first week of life, (b) other pathogens (other than E. coli) involved in neonatal diarrhoea and the diagnosis of the causes of diarrhoea and mortalities, (c) possible effects of the environment (cold weather during the study), management policies and other factors involved in a actively producing farm (blinded and equal treatment of diarrhoeic animals with sulphonamide-trimethoprim and gentamycin sulphate on both groups) to minimize production losses, and (d) dam effects (variable milk quality and quantity).

CONCLUSION

In conclusion, this pilot study showed significant reduction in the first week mortality of neonatal piglets and Day-one diarrhoal percentages (p < 0.05) in piglets from gilts vaccinated with E. coli (Neocolipor – Merial) vaccine. The result is reproducible as it is in agreement with other field trials (Anon, 2003) which indicate that E. coli vaccination in dams could be an alternative way of moderating mortality due to E. coli in a farm environment. No significant differences (p > 0.05) were observed in the overall diarrhoeal percentages and growth parameters at the other ages monitored. These could be attributed to various factors, i.e.: (1) unavailable data after the mortalities of piglets, in particular from the Control group, (2) this study had no real control over external factors (ambient temperature, environmental stressors and routine farm practices such as giving treatment to the diarrhoeic piglets). More field trials and experimental studies are warranted to further investigate the effects of E. coli vaccination in gilts as well as on sows under natural and controlled environmental conditions to show its benefits on the control of piglet diarrhoea and subsequent performance of pigs in farm environments in Malaysia.

CONFLICTS OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

We are grateful to our collaborator, Universiti Putra Malaysia for availing the results to be presented here. Partial funding and technical cooperation was provided by the farm involved in this study. We are grateful to acknowledge the technical assistance of Chow Guo Hao, Vania Kiu Tse Ling, Dr Ch'ng Chee Keong and also a few respectable personnel working in the farm during the course of this study.

REFERENCES


OCCURRENCE OF SALMONELLA AND OTHER ENTERIC MICROBES IN FAECES OF
HOUSE LIZARDS (Hemidactylus frenatus)

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SUMMARY

Reptiles have been shown to be natural reservoirs of Salmonella and other enteric bacteria and the reptile species close to homes and eateries are house lizards. This study aimed to determine the occurrence of Salmonella in house lizards at residential and eateries premises. Fresh faecal samples were collected from live 25 house lizards and 20 pooled dried droppings were collected around premises. None of the samples were positive for Salmonella. It was probable that a number of lizards may be carrying Salmonella as shown by other previous studies and in this case they were not shedding the bacteria in the faeces at the time and in the dried faecal droppings, Salmonellae was probably absent or did not survive the dry and hot condition. Enteric bacteria that were frequently isolated from fresh droppings were Klebsiella pneumoniae and Citrobacter freundii which were found resistant to amoxicillin + clavulanic and tetracycline.

Keywords: Salmonella, enteric bacteria, house lizards / geckos, antibiotic resistance

INTRODUCTION

Salmonella lives in the intestines of vertebrates and has been frequently reported in herpatofauna, particularly reptiles. The presence of Salmonella in reptiles was first reported in Gila monster and the regal horned lizard (Chambers et al, 2006). The increase in popularity of exotic reptiles, such as turtles, snakes and iguanas, as pet animals has led to increase in reptile-associated salmonellosis, such as in United States, which is estimated at 93,000 cases annually (Schroter et al, 2004; Pasmans et al, 2005). One reptile species that is of interest is the house lizards or geckos because they are a common sight in residential, commercial and eateries premises. These house lizards are native to Southeast Asia but are now invasively distributed worldwide in other tropical regions and also subtropical regions such as northern parts of Australia, Africa and America. They can be seen at night on walls and ceilings of houses, buildings, eateries, porches and balconies to hunt for insects that are attracted to lights.

Salmonella presences in reptiles are often asymptomatic and they shed the organisms, continuously or intermittently, in the faeces. Humans and pet animals may be infected if they are in contact with Salmonella-laden faecal materials. Conditions associated with salmonellosis in humans include gastroenteritis, bacteraemia, meningitis, osteomyelitis, peritonitis and pleuritis (Chambers et al, 2006).

Salmonella are ubiquitous in nature and able to survive for weeks in water and for years in soil and dust in environmental conditions (such as temperature, humidity and pH) that are favourable (Todar, 2006). Thus, the bacteria can survive in dried faeces in the environment for a period of time. However, the occurrence of Salmonella in the faeces was low compared to geckos that were actually carrying the bacteria (Chan et al, 1982).

Nonetheless, these faeces or droppings are still considered as route of transmission of the infection. Little is known on the faecal carrier status of Salmonella in the house lizards. Apart from Salmonella, not much is known regarding the occurrence of other pathogenic microorganisms in their faeces. This is because the droppings may contaminate food or water supply or the premises environments and that humans may be in contact with these faeces. Thus, the aim of this study was to determine the occurrence of Salmonella and other enteric microbes in the faeces of house lizards and the antibiotic resistance of the bacteria isolated.

MATERIALS AND METHODS

Samples collection

The premises identified to trap the live house lizards were two blocks of a residential college and two cafeterias in UPM, an apartment block and a water tank on the highest floor of an apartment building.

A total of 25 live lizards were caught. These lizards which crept on ceilings and walls were swept down and then captured using gloved hands. The lizards were then individually placed in a bottle (5L) containing a small box in which the lizard can hide inside. The lizards were kept until they defecated. Fresh faeces were collected within 12 hr after defecation using a sterile glove, placed in a plastic bag and brought to the Veterinary Public Health Laboratory, UPM. Most of the lizard defecated within a day after they were captured but a small number took two days. Those that did not defecate within two days were fed with a meal worm until they defecated. Once the faeces were collected, the lizard was released.
Twenty (20) pooled lizard droppings were collected from different residential and eateries premises. The lizard droppings could not be differentiated physically in terms of age but were estimated to be more than 5 - 7 days old. Ten (10) of the droppings were collected from several eateries in Seri Kembangan and the other 10 were collected from various houses in Serdang. These droppings could be found on walls, furniture tops, window panes and corners and edges of rooms. The droppings on the floor were not collected.

**Bacteria culture, isolation and identification**

Unlike fresh faeces, isolation of enteric microbes was not done on the dried droppings, that is, isolation of *Salmonella* was only conducted on the dried faeces. Each faecal dropping was added into 10ml of buffered peptone water in a bottle for pre-enrichment. The mixture was incubated at 37°C for 24 hr. In the enrichment stage, 1ml of the preenriched sample was transferred into Rappaport Vassiliadis Enrichment Broth (Oxoid). The mixture was then incubated at 37°C for 24 hr. After incubation, a loopful of the enriched culture was inoculated onto Brilliant Green Agar (BGA) and Xylose Lysine Tergitol 4 (XLT4) agar (Oxoid) and the plates were incubated at 37°C for 24 hr. Then, the plates were examined for bacterial growths and colonial morphology and the cellular morphology was examined by Gram staining. Different colonies were subcultured onto the same agar for purification. On BGA, typical *Salmonella* colonies appeared red whereas on XLT4, the bacteria colonies appeared black or black-centred with a yellow periphery. The suspected *Salmonella* colonies from pure cultures were subjected to biochemical tests for identification which consisted of inoculation into Triple Sugar Iron agar, Lysine Iron agar, Sulphide Indole Motility agar and subjected to citrase and urease tests. Then, suspected *Salmonella* colonies were subjected to a serological test, that is, a slide agglutination test using polyvalent O *Salmonella* antisera. To isolate other enteric microbes, the faecal samples were cultured on Blood agar (Oxoid) and MacConkey agar (Oxoid) to isolate Gram positive and Gram negative bacteria respectively. All the different types of colonies obtained were subjected to appropriate biochemical tests to identify the Gram positive and Gram negative bacteria as described in Jang et al (2008).

**Antibiotic susceptibility test**

Bacteria that were found predominant in the lizards caught in each premises were subjected to antibiotic susceptibility test using the disc diffusion method of Kirby Bauer as described by NCCLS (2002), now known as Clinical and Laboratory Standards Institute or CLSI. Six (6) antibiotics were used, namely streptomycin (S, 10µg), chloramphenicol (C, 30µg), amoxyccilin + clavulanic acid (AMC, 30µg), tetracycline (TE, 30µg), trimethoprim-sulfamethoxazole (SXT, 25µg), and enrofloxacin (ENR, 5µg). Briefly, each isolate was suspended into 2mL nutrient broth and standardized to 0.5 Mac Farland standard. A sterile swab was dipped into the suspension and swabbed over the entire surface of Mueller Hinton (MH) agar (Oxoid) plates in three overlapping directions - horizontally, vertically and obliquely. The plates were allowed to dry for approximately 5 min. The six antibiotic discs were dispensed onto the inoculated MH agar plate by using an antibiotic disc dispenser. After incubation, the plates were examined against a dark background to clearly visualize the zone of inhibition around each antibiotic disc. The zone of inhibition was measured using a calliper. The measured zones were compared to the zone diameter interpretive standards breakpoints (NCCLS, 2002) or following the manufacturer instructions to determine the susceptibility of the isolates to the antibiotics.

**RESULTS AND DISCUSSION**

From the study, *Salmonella* was not isolated from all the fresh and dried samples. Nine (9) out of 20 dried droppings that were collected from various residential and eateries premises showed no bacteria growth. It is probable that the lizards may be carrying *Salmonella* but not shedding the bacteria in the faeces at the time, hence it would not be present in the sampled faecal droppings. This is because the study by Fazhana et al (2007) on 32 house geckos found 31.0% positive, with 43.0% and 28.0% from houses and eateries, respectively. In that study, *Salmonella* was isolated from the gastrointestinal tracts of the house lizards. A study by Calaway et al (2011) in Townsville, North Australia found 7% of the house geckos carried *salmonellae* in the large intestines. Otokune for et al (2003) reported *Salmonella* carriage rate of 32% in the gastrointestinal tracts of wall gecko (Geckoniidae) and 35% in pooled lizard droppings from various sources. A number of works had reported that *Salmonella* is able to survive in adverse environment for a considerable period of time; however in the dried lizard droppings sampled, *Salmonellae* was probably absent or did not survive the dry and hot condition. However, according to Otokune for et al (2003), *Salmonella* can survive longer in dry as compared to wet environments – 4 weeks in tap water and up to 6 and 8 weeks in droppings left exposed directly to air and mixed with dry sand respectively. The frequency of isolation of different species of enteric bacteria from the faeces of the house lizards sampled is shown in Table 1.

The predominant enteric bacteria from house lizards at the residential and eateries premises were *Klebsiella pneumoniae*, followed by *Citrobacter freundii*. Other bacteria that were isolated from the residential premises were *Klebsiella oxytoca* and *Proteus penneri* whereas from the eateries were *Enterobacter cloacae*, *Staphylococcus sp* and *Bacillus sp*. Apart from *Salmonella*, the enteric organisms which Gugnani et al (1986) isolated from wall geckos (*Hemidactylus brookei*) in Nigeria, included *Proteus*...
mirabilis, Pseudomonas aeruginosa, Escherichia coli, K. pneumoniae and E. cloacae. A number of these enteric bacteria are opportunistic pathogens that can cause infections to those who are immunocompromised such as the young, old, people with debilitating disease, cancer or Acquired Immunodeficiency Syndrome.

Figure 1 depicts the antimicrobial resistance among all the K. pneumoniae and C. freundii isolates. The two bacteria were found resistant to amoxicillin + clavulanic acid while 80% of K. pneumoniae isolates were resistant to tetracycline. The bacteria were susceptible to enrofloxacin, chloramphenicol, trimethoprim + sulfamethoxazole and streptomycin. The isolated bacteria showed low antibiotic resistance with no multiple drug resistance (resistant to four or more antibiotics) detected; it is most likely due to lack of exposures of the house lizards to environment that were contaminated with resistant bacteria (Pasmans et al., 2005).

The house lizards are a common sight in residential, commercial and eateries premises and they indiscriminately litter the premises with their droppings, also reported by Otokune et al (2003). Although in this study Salmonella was not isolated, other studies had shown otherwise. The presence of house lizards in close proximity to man and the enteropathogens they may carry could cause infections in man should man come in contact with enteropathogen-laden faeces or by contaminating food, water and the floors. A more detailed epidemiological study with larger sample size, more premises and wider locations and to include the zoonotic viruses and enteric parasites would paint a better picture into the possible role of the house lizards in sporadic zoonotic infections such as salmonellosis in households and eateries.

Table 1: Enteric microbes isolated from faeces of house lizards

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>No. of sample with isolates</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae</td>
<td>15</td>
<td>44.1</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>3</td>
<td>8.9</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>10</td>
<td>29.4</td>
</tr>
<tr>
<td>Proteus penneri</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>Staphylococcus sp</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>Bacillus sp</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>34</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

Figure 1: Antimicrobial resistance among Klebsiella pneumoniae and Citrobacter freundii isolated from house geckos
REFERENCES


FELINE HYPERTROPHIC CARDIOMYOPATHY – PREVALANCE, RISK FACTOR AND PATHOLOGICAL ASPECT

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Faculty of Veterinary Medicine, University Putra Malaysia, 43400 UPM Serdang, Malaysia

Hypertrophic Cardiomyopathy (HCM)

Prevalence of HCM

HCM is inherited as an autosomal dominant disease in both humans (Liu et al., 1993; Maron et al., 2003) and cats (Meurs et al., 2007; Meurs et al., 2005). It is the most prevalent cardiomyopathy disease in both species; however, several factors preclude accurate determination of the disease frequency. It has been estimated to affect 0.1 - 0.2% of the human population and is the leading cause of sudden cardiac death in adolescents (including competitive athletes) (Maron et al., 1995). In cats the prevalence is high and it is estimated close to 16% of the overtly healthy cat population are affected (Cote et al., 2004; Paige et al., 2009; Riesen et al., 2007a). A retrospective echocardiographic study reported that 57.5% of cats initially diagnosed as idiopathic cardiomyopathy (n = 106) were found to have HCM (Ferasin et al., 2003).

HCM is a disease of young-to-middle age cats between 8 months to 16 year (mean of onset around 6.5 year) (Kraus et al., 1999; Nakagawa et al., 2002). The youngest documented was 2 months old (Fujii et al., 2001) and was commonly observed in males (Fujii et al., 2001; Granstrom et al., 2011; Liu et al., 1981; Riesen et al., 2007a; Tilley et al., 1977), a pattern which is not seen in humans. Male cats were also observed predisposed to HCM earlier in life and with a more aggressive development of the disease (Atkins et al., 1992; Rush et al., 2002), compared to female cats.

Generally, cats are a sedentary animal by nature but stressful situations (e.g. cat fights or chased by dog – increased sympathetic activity of the heart), may induce sudden death in cats with asymptomatic HCM (Kittleson and Kienle, 1998). A cross-sectional echocardiographic study identified a high frequency of 8 - 10% HCM amongst overtly healthy cats (Riesen et al., 2007a), which suggested that substantial changes to the heart were apparent well before any clinical signs were observed. This asymptomatic incidence of HCM partially explains episodes of sudden death or aortic thromboembolism that occurs in apparently young healthy cats without any obvious clinical signs (Baty et al., 2001; Cote et al., 2004; Liu and Tilley, 1980; Riesen et al., 2007b). Two large retrospective studies reported that the median survival time for cats presented with aortic thromboembolism, congestive heart failure and asymptomatic cats were between 2 to 6 months, 6 to 18 months and 3 to 5 years, respectively (Atkins et al., 1992; Rush et al., 2002).

Genetic factors of HCM

Abnormalities in the encoding sarcomeric proteins identified in HCM patients have led to a theory that it is a disease of contractile sarcomeric proteins (Marian and Roberts, 2001). HCM in humans is caused by a genetic mutation in one of the genes that encode for the sarcomeric proteins including β-myosin heavy chain (MyHC), cardiac troponin I and troponin binding protein C gene (MyBP-C) (Marian and Roberts, 2001; Maron, 2002). Other genes that accounted for a minority of human HCM cases were cardiac troponin I, regulatory and essential myosin light chain, titin, α-tropomyosin, α-actin and α-myosin heavy chain (Ommen and Nishimura, 2004). In human, HCM is inherited, usually as a heterogeneous autosomal dominant trait, in at least 2/3 of all HCM cases (Marian and Roberts, 2001; Solomon et al., 1990). To date, more than 1000 different mutations have been identified within 13 myofilament-related genes in human (Alcalai et al., 2008; Davies and Krikler, 1994; Solomon et al., 1990).

In cats, recent studies in Maine Coon and Ragdoll breeds have identified defects in the same sarcomeric protein genes, MyBP-C, but in different locations (Kittleson et al., 1998; Meurs et al., 2008; Meurs et al., 2007; Meurs et al., 2005), heritable in an autosomal dominant pattern (Baty and Walkins, 1998; Fananapazir and Epstein, 1994; Kittleson et al., 1999; Meurs et al., 2005). In addition to these breeds, there is anecdotal evidence of the familial heritability of HCM in other breeds, including Persian, British Shorthair, Norwegian Forest Cat, Turkish Van, Scottish Fold, Siberian, Sphynx and others (Granstrom et al., 2011; Meurs et al., 2009; Tilley et al., 2008), although the exact genetic cause in these breeds have not been well studied. The familial nature of HCM has also been reported in domestic (mixed-breed) cats (Kraus et al., 1999; Nakagawa et al., 2002). A long term observation on the progression of HCM in a family of domestic shorthair cat has been documented (Baty et al., 2001).

HCM in Maine Coon cats closely resembles human familial HCM in terms of mode of inheritance, phenotypic expression and disease course (Fananapazir and Epstein, 1994; Kittleson et al., 1999; Meurs et al., 2005). Recent study have found that the MyBP-C gene mutation in Maine Coon cats is breed specific and may not appears to be

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associated with the familial HCM in other breed of cats. The mutation is inherited with incomplete penetrance (heterozygous) with variable expressivity where not all cats with the mutation will show the disease or the severity of the disease may varies among cats (Figure 1) (Carlos Sampedrano et al., 2009; Lyons, 2010; Meurs et al., 2005). Carlos Sampedrano et al. (2009) identified 18% of Maine Coon cats (n = 8/44) with MyBP-C has HCM, hence not all cats with the identified gene developed HCM. The actual prevalence may have been underestimated and probably a long-term follow-up would accurately establish the onset of disease and its effects on all ages of cats. It was also suggested that there are likely more than one mutation responsible for HCM in this breed (Carlos Sampedrano et al., 2009). In Ragdoll cats, the substitution mutation identified in the MyBP-C, differs from the Maine Coon cats because the mutation is located in a different region. Therefore, Maine Coon and Ragdoll cats’ mutations were unlikely inherited from a common ancestor. The mode of inheritance in Ragdoll cats is yet to be identified and this breed-specific mutation has not been identified in other breeds of cats. In Ragdoll cats, it was shown that the homozygous cats appeared to be very severely affected, often before 2 years of age and the heterozygous cats appeared to have a milder form of the disease (Meurs et al., 2007).

In summary, the identification of the first sarcomeric gene mutation, MyBP-C in Maine Coon cats is highly significant and supports a role for cats as an alternative to transgenic mice as a animal model of human familial HCM (Figure 1) (Baty, 2004; Baty and Walkins, 1998; Hasenfuss, 1998).

![Diagram](https://example.com/diagram.png)

**Figure 1: Example of a colony of cats with familial HCM [an autosomal dominant inheritance with incomplete penetrance (heterozygous)]**

**Pathogenesis and progression of HCM**

It is a primary disorder of the myocardium characterised by concentric left ventricular hypertrophy, can be further described as mild-to-severe (thicken wall of a normal-to-small chamber size) (Kittleson and Kienle, 1998). To distinguish either: mild hypertrophy from normal, mild-to-moderate hypertrophy, severe hypertrophy or secondary due to other abnormalities are often not easy (Boon, 1998; Fox et al., 1999; Moise et al., 1986). HCM is phenotypically heterogeneous and no single distribution of left ventricular hypertrophy is typical of the disease (Kittleson and Kienle, 1998). Many have compared and correlated echocardiography findings with other diagnostic methods such as electrocardiography, necropsy, histopathology and computed magnetic resonant imaging to correlated the site or severity of hypertrophy and clinical findings (Fox, 2003; Sato et al., 1998).

HCM consist of two forms; obstructive and non-obstructive. Hypertrophy obstructive cardiomyopathy (HOCM) occurs when the hypertrophied basilar interventricular septum impinges and results in narrowing of the left ventricular outflow tract. The mitral valve is pushed into the outflow tract causing systolic anterior motion (SAM) of the mitral valve causing further obstruction of the outflow tract. At the onset of SAM of the mitral valve in HCM patients, the pushing force of flow is caused by the dominant hydrodynamic force (Sherrid et al., 2000), although the Venturi force are present in the outflow tract but does not contribute to the mechanism of SAM. Hence, the obstruction may be either dynamic, fixed or both (Boon, 1998; French, 2008; Takemura et al., 2003). SAM of the mitral valve is infrequently reported in cats with HCM but one isolated report by Fox et al. (1995) found that SAM was a common abnormality identified in 67% of the HCM cats (n=46). However, this condition may not present in all HCM cats and some cats may develop this condition before any evidence of wall thickening or some cats may have SAM of the mitral valve as a primary disease (Fox et al., 1995; Kittleson and Kienle, 1998; Klues et al., 1993) and similar has been widely studied and reported in HCM patients (Klues et al., 1993; Spirito and Maron, 1984).
Therefore, it is important to distinguish between obstructive and non-obstructive forms of HCM, as clinical decisions depend on the presence or absence of outflow obstruction (Kittleson and Kienle, 1998). SAM of the mitral valve produce two abnormalities where: (i) it obstructs blood flows out of the left ventricle in systole (dynamic sub-aortic stenosis) causing an increase blood flow velocity through the sub-aortic region producing a turbulence and; (ii) the septal leaflet drawn out from its normal position creates a mitral regurgitation due to SAM (Kittleson and Kienle, 1998). Dynamic outflow tract obstruction if severe will be a stimulus for concentric hypertrophy and potentially worsen left ventricular diastolic dysfunction. Hence, coupled with other structural pathological change (i.e. myocardial fibre disarray, myocardial fibrosis), it may exacerbate or potentially accelerate progression to heart failure.

Gross pathology

Cats with severe HCM have severe thickening of the left ventricular myocardium [the interventricular septum (IVS) and left ventricular free wall (LVFW)], papillary muscle hypertrophy and left atrium enlargement with possible thrombus present. The left ventricular chamber is smaller than normal due to the inwards myocardial thickening into the left ventricular cavity (Cesta et al., 2005; Fox, 2003; Liu et al., 1981; Liu et al., 1993; Tilley et al., 1977). The distributions of the myocardium thickness in most cases may not be the same. The IVS and the LVFW are equally thickened (symmetric hypertrophy) in most cats with severe HCM. Other cats may have significantly thicker IVS compared to the LVFW or vice versa. Cats with mild-to-moderate HCM have lesser myocardial wall thickness, hypertrophy papillary muscle and probably a normal size of left ventricular and atrium chamber (Fox et al., 1999; Kittleson and Kienle, 1998; Tilley et al., 2008). Heart weight was reported to be a useful indicator for disease severity or to identify hearts with hypertrophy grossly. The heart weight in relation to the body weight has been reported to be 4.8 ± 1.8 g/kg in healthy cats versus 6.0 ± 1.4g/kg in cats with HCM (Fox et al., 1995).

Histopathology

HCM has a wide range of histopathological abnormalities but myocardial fibre disarray is a distinctive hallmark of HCM. The myocytes are arranged in chaotic, disorganised patterns at oblique and/or perpendicular angle which appeared in a bizarre disorganised cellular architecture (Baty et al., 2001; Fox, 2003; Kittleson and Kienle, 1998; Liu et al., 1993; Nakagawa et al., 2002; Tilley et al., 1977). Other histopathological findings are hypertrophied myocytes, increased collagen deposition resulting interstitial fibrosis and abnormalities of the intramyocardial small vessels (Cesta et al., 2005; Fox, 2003; Liu et al., 1993; Nakagawa et al., 2002; Varnava et al., 2000). It is likely that these abnormal structural changes disrupt the transmission of electrophysiology impulses predisposing the diseased heart to diastolic dysfunction and ventricular tachyarrhythmia (Fox, 2003; Kittleson and Kienle, 1998; Liu et al., 1981; Liu et al., 1993; Tilley et al., 1977). The frequency of each abnormality observed varied between HCM cats (Liu et al., 1993). Liu et al. (1981) found 25% of the HCM cats (n=51) observed with asymmetric left ventricular hypertrophy had myocardial fibre disarray in the IVS. In other HCM hearts, only myocyte hypertrophy was evident or some had moderate-to-severe interstitial. Replacement fibrosis was present in about 20 - 40% of HCM cases (Liu et al., 1993). The histopathological findings differ between HCM hearts probably due to cardiac remodelling which occurs at different stages of the disease, maybe depending on the extent of the damage. Hence, whether the asymptomatic cats with HCM have similar histopathological findings at the early stage despite detectable functional changes is unknown and warrant investigation.

Pathophysiology

The pathogenesis of HCM in both humans and cats, to our knowledge is still not fully understood. It is a known genetic cardiovascular disease due to the mutations in genes encoding proteins of the cardiac sarcomere, but the molecular pathogenesis that leads to the development of hypertrophy and the variability in the common pathological phenotypes expression still remain unknown (Abbott, 2010; Braunwald et al., 2012; Lind et al., 2006; Marian, 2000; Maron et al., 2009). A mouse model has recently been used to gain greater understanding of this complex genetic disorder (Berul et al., 2001; Geisterfer-Lowrance et al., 1996; James et al., 1998; Prabhakar et al., 2003; Shephard and Semsarian, 2009; Welikson et al., 1999) as studies of molecular and pathophysiological mechanisms in cats or human HCM patients is difficult, particularly since the disease is often well established before diagnosis, especially
in cats. Pathophysiological changes and/or dysfunction presence at the early stage of HCM in cats well before the clinical sign was observed but actual mechanism is still widely debated. The genetic mutation or the pathophysiological mechanism is a trigger of the HCM is still unknown, to our knowledge.

What is understood now are that in any particular HCM cat, one or more of the pathophysiological changes and dysfunctions may occur including: (i) left ventricular diastolic dysfunction; (ii) ventricular and supraventricular tachyarrhythmias; and (iii) myocardial ischemia and fibrosis (Kittleson and Kienle, 1998; Maron et al., 2009; Tilley et al., 2008). Coupled with those important derangements, there are other under-recognised emerging pathophysiological concerns such as the possible occurrences of myocarditis (Bayes-Genis, 2007) and autonomic nervous dysfunction (Morner et al., 2005) to our knowledge has not been given emphasis in cats with HCM.

**Left ventricular diastolic dysfunction**

The main functional implication in cats with HCM is diastolic dysfunction (Fox et al., 1999; Kittleson and Kienle, 1998; Tilley et al., 2008). The pathologcal changes of the concentric left ventricular hypertrophy from cardiac remodelling increased myocardial stiffness, increased end diastolic filling pressure and impaired early diastolic relaxation (Abbott, 2000; Fox, 2007; Liu et al., 1993).

In HCM, diastolic function is compromised by several mechanisms. Ventricular compliance is reduced as a result of left ventricular hypertrophy and small arterial changes that impair left ventricular perfusion, causing myocardial ischemia, necrosis and replacement fibrosis (Kitamura et al., 2001; Kittleson and Kienle, 1998). Compliance is further compromised by myocardial fibre disarray which has been shown as the most important factor related to diastolic dysfunction in humans HCM (Osato et al., 1989) but assumed to contribute similar consequences in cats with HCM (Kittleson et al., 1999). Besides that, impaired sarcoplasmic calcium channel regulation and impaired calcium uptake by the sarcoplasmic reticulum leads to increased intracellular calcium concentration and impaired active relaxation (Gwathmey et al., 1991; Opie, 2004).

**Ventricular and supraventricular tachyarrhythmias**

Correlation between left ventricular hypertrophy and arrhythmogenic sudden death is well established not only in HCM, but in other conditions that contribute to left ventricular hypertrophy (i.e. hypertensive and aortic stenosis) (Douglas and Tallant, 1991; Piorecka-Makula and Werner, 2009; Wolk, 2000). The most consistently observed abnormality that predisposed to arrhythmia is early or delayed afterdepolarisation and triggered activity leading to prolong action potential durations and refractoriness. In addition, non-uniform prolongation of the action potential may lead to increased dispersion of repolarisation or refractoriness and favouring re-entry in the heart (Levick, 2003; Strickland, 1998; Wolk, 2000). In humans with HCM, the disorganised cellular architecture, myocardial fibrosis and scarring due to cardiac remodelling has been suspected as an arrhythmogenic substrate predisposing to the life-threatening electrical instability (Marian and Roberts, 2001; Ommen and Nishimura, 2004; Spirito et al., 1987).

Spirito et al. (1987) documented a strong association of severe-to-moderate left ventricular hypertrophy with significantly increased occurrences of ventricular tachycardia in HCM patients. Cats with a history of episodic collapse or dyspnoea diagnosed with HCM were identified with a high frequency of severe ventricular arrhythmias (i.e. ventricular premature complexes, ventricular bigeminy, ventricular tachycardia, supraventricular tachycardia, supraventricular premature complex) (Bright and Cali, 2000; Ferasin et al., 2003; Goodwin et al., 1992).

**Myocardial ischaemic and fibrosis**

Application of positron emission topography and cardiovascular magnetic resonant has allowed evaluation of active myocardial ischemia as a determinant of progressive heart failure in human HCM patients (Harris et al., 2006; O’Gara et al., 1987; Olivotto et al., 2004). The combination of increased left ventricular wall thickness (increased myocardial oxygen demand) and decreased capillary network (decreased myocardial oxygen supply) will increase heart rate and afterload, while decreased perfusion may predispose to myocardial ischemia (Ommen and Nishimura, 2004).

Presence of myocardial ischemia due to microvascular dysfunction in HCM has been suggested as an important pathophysiological component of the disease progression (Maron et al., 2009). Cats with HCM have coronary remodelling (arteriosclerosis or “small vessel disease”) similar to that described in humans with HCM (Baty et al., 2001; Nakagawa et al., 2002; Takemura et al., 2003). Liu et al. (1993) identified intramural coronary arteriosclerosis in 75% (38/51) of cats diagnosed with HCM. Both, Varnava et al. (2002) and Liu et al. (1993) described a common finding and relate that the intramural coronary arteriosclerosis are particularly prominent in tissue sections with moderate-to-severe fibrosis. There may therefore be a relationship between arteriosclerosis and myocardial fibrosis. With compromised blood flow in small vessels triggering myocardial ischemia, with subsequent cell death (necrosis) and scarring (fibrosis) affecting the clinical course of the disease.

Varnava et al. (2000) proposed that myocardial fibre disarray was a direct response to the functional and structural abnormalities of the mutated sarcomeric protein, although the authors also considered that fibrosis and small vessel disease were secondary response unrelated to disarray. Myocardial ischemia has been linked to
ventricular tachycardia and sudden death (Wolk, 2000) and its presence in the early stages of HCM may actually be a pathogenic factor.

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THE ROLE OF OMEGA-3 POLYUNSATURATED FATTY ACIDS ON BRAIN COGNITIVE FUNCTION - REVIEW OF STUDIES ON LABORATORY ANIMALS

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OVERVIEW

Fatty acids are essential components of the diet and sources of food energy. A study carried out by Burr and Burr, (1929) first acknowledged that specific components of fatty acids may be necessary for the proper growth and development of animals and humans. They proposed that clinical signs of essential fatty acid (EFA) deficiency was correlated with cardiovascular disease and compromised immune system. In mammals, the fatty acids have been recognized as important nutrition factors for brain cognitive performance. Nutrient supplementation such as omega-3 ($\omega$-3) fatty acids are widely accepted to improve diets, and contribute to the maintenance of good health, especially on the cardiovascular system and other aspects of health, such as cognition throughout life. Fish oil from tuna and salmon are the richest source of docosahexaenoic acid (DHA) that affects the cognitive function of the brain. This could indicate why fish has been called "brain food" and why DHA deficiencies can have a life-long impact on intelligence. Thus, knowledge of health benefits fatty acid as a dietary trend to increase good health status. In fact, world health organizations such as the UK Food Standards Agency and the Japan Society for Lipid Nutrition, advise that more people should aim to increase their intake of $\omega$-3 fatty acid supplementation. As these fatty acids have an essential role during brain development, the first section of this review examines the importance of $\omega$-3 fatty acid in relation to the metabolism. This will be followed by a review of the role of fatty acids on spatial recognition memory such as neural membrane function, cholinergic neurotransmitter and animal behavior studies. Experimental works in mice and rats have shown that $\omega$-3 fatty acid supplementation can increase levels of synaptic vesicles, neuron dendrites and cholinergic neurotransmitters. The mechanism physiological of brain gene expression and cognitive function by $\omega$-3 fatty acid also will be examines in this section. Thus, this review is focused on dietary $\omega$-3 fatty acid supplementation on brain cognitive function and the mechanism interaction between $\omega$-3 fatty acid and genetic functions on laboratory animals.

Fatty acids

Fat is a substance containing one or more fatty acids bound to a glycerol backbone (Rudin and Felix, 1996; for review see Sprecher, 2000). Fats play major roles in the metabolic, storage and protective functions of the mammalian body (Gur and Harwood, 1991). Practically, fatty acids can be from four to twenty-eight carbons in the chain, with can be classified as short-, medium-, or long-chained. In addition, fatty acids can also be classified as saturated fatty acid (SFA) and unsaturated fatty acid (UFA) (Rudin and Felix, 1996). In SFA, the carbons in the chain are completely saturated with hydrogen atoms. The results are a dense and solid fat such as the white fat in beef and lamb produced butter that does not melt at room temperature (Rudin and Felix, 1996). However with UFAs, especially polyunsaturated fatty acids, the carbons carry less hydrogen.

Polyunsaturated fatty acids (PUFAs) are essential for normal growth and development. The PUFAs are classified according to the position of the first double bond from the methyl terminal end. The first double bond in $\omega$-3 is found at the third carbon atom from the methyl terminal, whereas in $\omega$-6 the first double bond is located after the sixth carbon atom from the methyl terminal. The precursors of two families of PUFAs namely, linoleic acid (LA, $\omega$-6) and $\alpha$-linolenic acid (ALA, $\omega$-3) are termed essential because they are cannot be produced by the animal or human body and must be supplied from the diet (Birberg-Thornberg et al., 2006). Sources of $\omega$-6 PUFAs and LA are found mainly in vegetable products such as soybean, corn, nut, and sunflower oils (Bouziane et al., 1992; Madani et al., 1998). However, sources of $\omega$-3 fatty acids are based on fish (menhaden, mackerel, herring, and salmon) and vegetable (rapeseed, soybean and nut) oils (Aid et al., 2005). However, the ALA is also found in the chloroplast of green leafy vegetables, such as spinach, seeds of flax and linseed (Kitessa et al., 2003).

A significant proportion of the fatty acids are present as PUFA derivatives of the two parent essential fatty acids, ALA and LA. The precursor essential fatty acids ALA and LA are metabolized by a process of desaturation and chain elongation. These precursors undergo sequential desaturation through the addition of double bonds and elongation by addition of carbon atoms. The DHA is synthesized from ALA by the addition of a double bond by a $\Delta$6-desaturase to form stearidonic acid (SDA, C18:4$n$-3). The elongation of SDA forms eicosatetraenoic acid (ETA, C20:4$n$-3) and the addition of another double bond by a $\Delta$5-desaturase produces eicosapentaenoic (EPA, C20:5$n$-3). The elongation of EPA forms docosapentaenoic (DPA, C22:5$n$-3), and the final addition of a double bond produces DHA (for review see Sprecher et al., 1995). The long chain $\omega$-6 fatty acid synthesis such as the elongation of LA to DPA

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occurs via the same alternating desaturation and elongation steps.

Role of ω-3 fatty acids on spatial recognition memory

ω-3 fatty acids and neural membrane function

ω-3 fatty acids are the major structural components of membrane phospholipids (Bertrand et al., 2006). They influence membrane fluidity and ion transport across cell membranes (Ehringer et al., 1990). Among the long chain fatty acids, DHA is an important structural component of the highly lipid biomembrane of neuron cell (for review, see Lauritzen et al., 2001). Previous studies report that DHA deficiency caused a reduction in the size of neurons of the brain region in the hippocampus (Ahmad et al., 2002). These authors showed that neuron size in the hippocampus, hypothalamus and parietal cortex are decreased in rats which are fed a DHA deficient diet containing 1.9gm/100gm safflower oil compared to those fed on a DHA adequate diet (Flaxseed oil, 0.48gm/100gm). The authors also reported that the brains of these rats exhibited a nearly 90% decrease of DHA. Thus, these results indicate that membrane neuron cell depends on DHA content for giving an optimal function in neurons such as signal transduction and synaptic activity.

Further evidence that ω-3 fatty acids promotes neurite growth in hippocampal neurons comes from the previous works reporting that DHA supplementation of about 2.6% in culture increased the population of neurons with longer neurite length per neuron and with a higher number of branches (Calderon and Kim, 2004). The author suggested that rats containing a lower level of DHA (about 0.1%) showed decreased neurite length, branches and neuron population, and consequently impaired their cognitive performance. This was supported by previous studies reporting that the DHA promotes neurite growth and survival in hippocampal neurons (Cao et al., 2005). These findings suggest that an ω-3 fatty acid family, especially DHA, selectively promotes the development of hippocampal neurons, which in turn affects the number and quality of synaptic connections during brain function.

Another approach valuable for the evaluation of the role of ω-3 fatty acids comes from the previous works reporting that an ω-3 fatty acid sufficient diet can promote structural changes in hippocampal neurons (Yoshida et al., 1997). The studies explained that rats with an ω-3 fatty acid deficient diet containing safflower oil show a 30% decreased density of synaptic vesicles in the terminal of the brain region compared with those in the DHA group (perilla oil). These results suggest that an ω-3 fatty acid deficient diet decrease lead to synaptic vesicle functions such as synthesis movement to releasable sites and fusion with synaptic membrane in the hippocampal region. Additionally, ω-3 fatty acid supplementation increased the number of synaptic vesicles (Weisinger et al., 1995) and improved fluidity of the synaptic membrane which improved interneuron communication and signal transduction (Ahmad et al., 2002; Calderon and Kim, 2004; Yoshida et al., 1997). Functionally, the fluidity of neuronal membranes affects the signal processing properties of neurons and can improve neural performance (Ehringer et al., 1990). Overall, it can be suggested that ω-3 fatty acid is essential in normal neurogenesis and synaptogenesis, and is also linked to improvement in learning and memory function.

ω-3 fatty acids and cholinergic neurotransmitter

Acetylcholine (ACh) is a neurotransmitter which is found in both the peripheral nervous system and central nervous system (CNS) in many organisms (Mathew et al., 2007). This neurotransmitter is released from the brain neurons to extracellular fluids and plays important roles in various biological processes such as cognitive functioning, memory and emotion (Zhang et al., 2002). In addition, central cholinergic activity facilitates human and animal cognitive function (Harmon and Wellman, 2003). A disturbance in the central cholinergic systems such as decreased ACh levels are partly responsible for the decline in cognitive functions in Alzheimer’s patients (Bennett et al., 2007) and aging humans (Leung et al., 2003). Previous studies report that cholinergic activity facilitates long-term potentiation in various areas of the brain such as cerebral regions and cerebrospinal fluid levels of choline and acetycholine (Dash et al., 2007). Thus, this could indicate that high level of cholinergic neurotransmitter such ACh is important for the improvement cognitive and memory functions.

It is known that ω-3 fatty acids can modulate the ACh in the brain by diet with ω-3 supplementation in rats (for review, see Young and Conquer, 2005). Previous studies report that an increase ACh cerebral levels following administration of dietary 5% DHA are correlated with an improvement performance in passive avoidance tasks in a rats (Minami et al., 1997). This theory is supported by the finding that ω-3 fatty acid sufficiency from tuna oil acts to enhance the stimulated synaptic release process of ACh in the hippocampus, which consequently contributes to the improvement of learning and memory performance in rats (Aid et al., 2005; Aid et al., 2003). This could indicate that ω-3 fatty acid plays a key role in ACh function via changes in the brain phospholipids composition and may consequently improve learning and memory function.

A previous study reported that the behavioral and cognitive changes in rats induced by ω-3 fatty acid deficiency could be due to changes in cholinergic neurotransmitters (Delion et al., 1994). Similarly, cholinergic neurotransmission in the hippocampus is specifically affected by a diet-induced lack of neuronal ω-3 fatty acid (Aid et al., 2003). Furthermore, there may be functional interactions between cholinergic and monoaminergic systems that are altered by an ω-3 fatty acid
deficient diet (Delion et al., 1994). Both systems contribute to the impairment of hippocampal function and induce behavior performance disturbances in rats. Thus, the hippocampal cholinergic system plays a major role in the regulation of cognitive functions and its modification might contribute to the cognitive and behavioral disturbances that occur in ω-3 fatty acid deficient diet.

**ω-3 fatty acids and cognitive functions**

A diet with ω-3 fatty acid especially DHA maintains a fluid synaptomic membrane and consequently improves the ability of learning and memory function in mammals (Suzuki et al., 1998). Previous studies have shown that ω-3 fatty acid deficient mice demonstrated impaired learning in the memory version of the Barnes circular maze as they spent more time and made more errors in search of an escape tunnel (Fedorova et al., 2007). In the brain’s fatty acid profiles, this study found a 51% loss of total brain DHA in mice with an ω-3 fatty acid deficient diet compared to ω-3 fatty acid sufficient mice. Similarly, previous studies report that the level of brain DHA decreased about 50% in mice on an ω-3 fatty acid deficient diet compared to control group on an ω-3 fatty acid sufficient diet (Carrie et al., 1999). These studies suggest that the ω-3 fatty acid deficient diet significantly decreased learning performance and retinal DHA level in adult mice. In a follow up study, the authors confirmed that an ω-3 fatty acid deficient diet altered fatty acid composition in brain regions and significantly reduced spatial learning as well as mice behavior (Carrie et al., 2000).

Further evidence that ω-3 fatty acid is required for cognitive function comes from the previous work reporting that ω-3 fatty acid deficient diet significantly decreased learning performance in adult mice (Umezawa et al., 1999). It was observed that mice which were fed on ω-3 fatty acid deficient diet consisting of 250.3g/100g safflower oil had significantly reduced learning performance compared with mice with ω-3 fatty acid sufficient diet (Perilla oil; 0.24g/100g). Deficiency of ω-3 fatty acid in the diet also leads to reduced brain DHA levels in rats (Moriguchi and Salem, 2003). The reduced levels of DHA lead to a loss in brain function as reflected in poorer spatial task performance. However, the spatial task performance of DHA deficient rats can be normalized after dietary ω-3 fatty acid supplementation for 13 weeks to restore brain DHA. Thus, this could indicate that learning and memory performance are correlated with the brain level of ω-3 fatty acid especially DHA.

Many studies have shown that ω-3 fatty acids from fish oil are essential for normal neurological development associated in learning and memory function (Carrie et al., 2000; Chung et al., 2008; Joshi et al., 2004). One example of such evidence comes from previous work reporting that a fish oil diet from sardine oil (100g/kg) induced a significant increase in exploratory activity and learning ability in young mice (Carrie et al., 2000). A similar effect suggested that adults mice which were fed on the sardine oil diet for a long period maintain higher levels of DHA brain phospholipids and improved learning ability (Suzuki et al., 1998). This was in line with the reports that fish oil (70g/kg; cod liver oil) supplementation during pregnancy improved cognitive performance in dams and their offspring (Chung et al., 2008; Joshi et al., 2004). In addition, fish oil supplements might reduce the risk of memory loss or Alzheimer disease in human populations (Cole et al., 2005). Overall, it is clear that dietary fish oil is important in neurological development associated with an improvement in brain cognitive function.

**ω-3 fatty acids and animal behavior task**

Previous studies have shown that there are several maze tasks that assess cognitive function including the Morris water maze (MWM), Y-maze and elevated plus maze (Abumrad et al., 2005; Astur et al., 2004). Dietary ω-3 fatty acid deficiency over three generations disrupted learning and memory performance in the MWM task by adult rats (Moriguchi and Salem, 2003). In the case of the MWM task, the ω-3 fatty acid deficient group showed longer escape latency and delayed acquisition of this task compared with the ω-3 fatty acid sufficient group. Previous studies report that rats with DHA (1%) and LNA (3.1%) dietary supplementation have a shorter escape latency in the MWM task compared to the rats in DPA (1%) and LA (1%) dietary supplementation (Lim et al., 2005; Lim et al., 2005). However, dietary fish oil induced a significant increase in exploratory and locomotor activity in the MWM test in young mice (Carrie et al., 2000).

Another approach valuable for evaluation of the role of ω-3 fatty acid in neuronal and cognitive function comes from the works reported previously (Blithe, 2005). This study demonstrated that mice were fed ω-3 fatty acid deficient diet containing peanut oil and the control-lipid diet containing a mixture of peanut and rapeseed oil (1200 mg LA and 300mg ALA per 100g diet). The spatial task performance of these animals was then compared using a two-trial recognition task in the Y-maze. The outcome of that study indicated that during the acquisition phase, the activity (number of visits/2/open arms) was the same whatever the dietary treatment administered. However, during the restitution phase, 45 min after the end of the acquisition phase or when the three arms were all open for exploration, animals in ω-3 fatty acid deficient group were less able to recognize the new arm compared to the mice fed the control-lipid diet. Based on these findings, it is quite evident that ω-3 fatty acid family (especially ALA) provided in the diet plays a key role in cognitive functions and is required for optimal brain function.

In the elevated plus maze, a test of anxiety, the time spent on open arms of the maze was significantly lower in ω-3 fatty acid deficient mice compared to the sufficient mice which received rapeseed oil 30g/kg and peanut oil 30g/kg (Carrie et al., 2000). Similarly, the time spent in open arms as well as the frequency of entry into the open arms tended
to be higher in the ω-3 fatty acid sufficient mice compared to deficient mice in the elevated plus maze of anxiety protocol (Nakashima et al., 1995). The findings of increased anxiety in ω-3 fatty acid deficient animals are supported by previous studies which also showed that ω-3 fatty acid deficient rats spent less time in the open arms compared to the sufficient group, signaling an anxiogenic response. However, after one week of supplementation with ω-3 fatty acid, the rats demonstrated a significant improvement in terms of the number of entries into the open arms. This condition may explain the improved behavior performance of anxiety protocol in the elevated plus maze as a result of the ω-3 fatty acid sufficient diet.

**Effect of ω-3 fatty acids on brain gene expression**

Fatty acid regulation of gene expression occurs in unicellular and complex organisms. Fatty acid also plays a role in controlling gene expression in a variety of tissues such as nerves and brain tissues (Barcelo-Coblijn et al., 2003). It has become evident that ω-3 fatty acid can also act as signaling molecules involved in regulating gene expression, eicosanoid synthesis and membrane structure (Duplus et al., 2000). Previous studies have reported that several genes are activated by dietary ω-3 fatty acid and some gene products have beneficial effects on brain functions such as learning and memory (Kitajka et al., 2002; Kitajka et al., 2004; Puskas et al., 2003). Further to this, DNA microarray technology is a good approach for identifying changes in transcription of multiple genes in certain brain regions such as hippocampus (Puskas et al., 2003) and dentate gyrus (Burger et al., 2007).

As has been reported previously, 55 genes were detected as overexpressed and 47 were suppressed in rats fed ω-3 fatty acid supplementation (Kitajka et al., 2004). These finding shows that the expression of genes can be altered by DHA supplementation, using cDNA microarray analysis. Several genes such as transthyretin participating in signal transduction processes were overexpressed in rat brains receiving a DHA-enriched diet for one month (Barcelo-Coblijn et al., 2003). Similarly, transthyretin gene was expressed by fish oil supplementation in a rat’s brain hippocampus over one month (Puskas et al., 2003). This gene binds thyroid hormones and plays an important role in cognitive function. In such studies, thyroid hormone deficiency during brain development impairs performance of cognitive function (Wilcoxon et al., 2007). This finding was similar in previous studies reporting that a low level of calmodulin-dependent protein kinase-II activation in a transgenic mouse model, resulted in enhanced performance in cognitive function which was associated with an increased transthyretin transcription (Butler et al., 1995). Thus, these could indicate that transthyretin gene has a marked influence in synaptic plasticity, learning and memory.

Further evidence that ω-3 fatty acid can modulate the suppression and enhancement of expression genes comes from the previous studies reporting that ω-3 fatty acid induces various genes involved in diverse functions in different brain regions (Kitajka et al., 2004). These findings proposed an experimental feeding protocol containing perilla oil, which is rich in ALA (39% ALA/29% LA) and fish oil rich in DHA (27% DHA/23% LA/3% ALA/12% EPA). This resulted in gene encoding synuclein α and γ which were over-expressed. These genes participated in signal transduction processes, synaptosomes and ion channel formation. In addition, synuclein possibly related to cognitive functions in young rats receiving ω-3 fatty acid from conception until adulthood (Barcelo-Coblijn et al., 2003). The synuclein is shown to accumulate in the brain of song birds during the period of song learning (Recchia et al., 2004). In has been shown that over-expression of synucleins appear to be associated with the development and maturation of neurons and neurotransmission (Eslamboli et al., 2007). Furthermore, genes participating in signal transduction processes such as calmodulins also were up-regulated by the dietary of high LNA (perilla oil; 8%) or high EPA + DHA (fish oil; 8%) (Kitajka et al., 2002). Interestingly, calmodulins may enhance communication between neurons during signal transduction process and have a special role in the stimulant-induced plasticity of the CNS (Jordan et al., 2007). Overall, dietary ω-3 fatty acid influences the transcription of key genes involved in cognitive function as well as being important for normal brain function and exerting protection against the incidence of neurodegenerative diseases such as Alzheimer disease.

**CONCLUSION**

Nutritional status is one of the factors that can influence learning and memory function in mammals. Food restriction is also increased the animal to lead learning and memory deficits. Increasing number of evidence shows that dietary ω-3 fatty acid in brain cell membranes is important to improve learning and memory function. In biochemistry study, ω-3 fatty acids also have important roles to regulate and modulate brain gene expression associated learning and memory function. Therefore, future study needs to address this issue especially dietary ω-3 fatty acid deficiency interacts with neural membrane function and cholinergic neurotransmitter associated with cognitive function. The mechanism by which ω-3 fatty acids modulate the gene expression associated with cognitive function is yet to be explored. It is important to understand the possible effect since this area of study is limited. Thus, a number of experiments need to be conducted to demonstrate the mechanism effects of ω-3 fatty acid in brain gene expression associated with cognitive functions.

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MODERATELY-HIGH HUMORAL ANTIBODY RESPONSES TO A H5N2 INACTIVATED VACCINE DID NOT SUPPRESS SHEDDING OF HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA VIRUS DURING CHALLENGE.


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SUMMARY

Twelve SPF chickens with moderately high-titred HI antibodies of $7 \log_2$ and $8 \log_2$ achieved at week 4 post-vaccination after a single vaccination with an inactivated whole-H5N2 virus vaccine developed at Veterinary Research Institute, Ipoh were selected for efficacy and shedding studies. The H5N2 vaccine virus shares approximately 88.2% homology to the HA gene of the H5N1 challenge virus. The chickens with moderately high titred HI humoral antibodies provided 100% protection against mortality and morbidity after challenged with a lethal highly pathogenic H5N1 Malaysian strain. The challenge H5N1 virus was reisolated from pooled cloacal swabs of chickens with HI titres of $7 \log_2$ and $8 \log_2$ at 3 days post challenged, however, was not reisolated from the pooled oropharyngeal swabs. The virus titre at reisolation was $10^3 \text{EID}_{50}/0.1 \text{ml}$ in pooled cloacal samples from both the $7 \log_2$ and $8 \log_2$ HI titred-chickens. There was no challenge H5N1 virus reisolated from chickens with HI titre of $9 \log_2$. This study demonstrated that chickens with moderately high HI humoral antibodies protect chickens against clinical disease and mortality did not fully prevent infection, however, was able to reduce virus shed via the cloaca and oropharynx.

Keywords: Avian Influenza, Virus, Vaccine

INTRODUCTION

The H5N1 epizootic outbreak has resulted in the increase in the vaccination against H5N1 in poultry population of countries such as China, Italy, Mexico, Pakistan and Indonesia. For these countries, vaccination seemed to be one of the principal means of combating highly pathogenic (HP) avian influenza, as vaccination can control infection and reduce the incidence of clinical disease, thus reduce viral load in the environment (Swayne and Suarez, 2007; Capua et al., 2007). As inactivated HP H5N1 vaccines are not feasible to be made into vaccines, due to reasons, such as incomplete inactivation which may result in disease and spread, and difficulty in differentiating from wild field and vaccine strains, inactivated vaccines based on reverse genetics and heterologous HA and NA antigens are therefore the best options. Inactivated vaccines, however, seemed to give variable results and boosters need to be given to achieve high immune responses. A study by Terrigino et al. (2006) showed that vaccination with an inactivated H5N9 subtype vaccine at 3 weeks and boostering at 7 weeks of age induced a very high immune response, GMT 10.3 $\log_2$. This immune response was protective against challenge with $10^6 \text{EID}_{90}/0.1 \text{ml}$ of the highly pathogenic A/chicken/Yamaguchi/7/2004 H5N1 subtype and suppress shedding after challenge. Studies in ducks and geese also showed variable results. A study on an inactivated reverse genetic (RG) vaccine H5N1/PR8 for ducks and geese, where the HA gene implicated in virulence is replaced with a non-pathogenic H5 gene, HI antibodies were only detectable at week 1 post vaccination (pv), and by 6 weeks pv, the antibody rose to a high HI titre of 1024 and waned off to a titre of 16 by 43 weeks (Tian et al., 2005). A bivalent inactivated vaccine of H5N9 +H7N1 and a monovalent H5N3 which was given to 1 day old and 3 weeks old ducks, induced only low titres of $2 \log_2$ - $3 \log_2$ and $3 \log_2 – 6 \log_2$ respectively, which was achieved at week 2 post vaccination. Despite the failure to stimulate significant HI titres, the bivalent vaccine did offer protection however, did not stop virus replication as seen in the seroconversion or the rise in antibody titres following challenge. The monovalent H5N3 vaccine, however, provided solid protection with no evidence of shedding of the challenge virus and no serological response to the H5N1 challenge virus (Middleton et al., 2007). Lee et al. (2007), however, showed that doses or quantity of antigen of the same HA subtype and boostering are important for protective efficacy of the vaccine against H5N1 challenge. In their study, they showed that one dose of 128 HAU and 64 HAU homologous H5 vaccine induced 100% and 50% protection respectively. Virus shedding was prevented with the 128 HAU but not with the 64 HAU antigen quantities. They also showed that two doses at a 3-week interval with 64 HAU as well as an extra one dose of 1024 HAU of heterologous H5N3 vaccine provided 100% protection and prevent viral shedding completely. This is in agreement with studies by Swayne et al. (1999) who showed that there was a correlation between the antigen quantity or antigenic...
content and protective efficacy of the vaccine. In contrast, others reported that high virus titres can still be present in vaccinated chickens that are protected against clinical disease (Maas et al., 2009) where in one study with H9N2 vaccine, after vaccination with as much as 128 or 1024 HAU low pathogenic (LP) H9N2 virus and despite the induction of high antibody titres, the LP H9N2 challenge virus could still be isolated from the vaccinated chickens (Choi et al., 2008).

The molecular and antigenic similarities of 96.8 - 100% between the individual H5 avian influenza strains were sufficient to elicit solid cross protection against emerging HP H5N1 viruses, however, there was no positive correlation between sequence identity and the ability to reduce the quantity of challenge virus shed. For example, a study by Kumar et al. (2007) showed that chickens with high HI titres (> 640) elicited by a reverse genetics H5N3 avian influenza isolate and challenged with the original H5N3 virus i.e 100% similarity in the HA genes, did not result in sterile immunity as virus can be reisolated from at least 1/16 chickens with high antibody titres (HI:160 - 640).

The purpose of the study was, therefore, to determine the protection and effect on challenge virus shedding by chickens with moderately high titre-antibody as induced by a heterologous whole-H5N2 virus vaccine strain, with an HA gene homology of 88.2% with the challenge H5N1 virus. The practical implication of this trial is that, if protection is afforded and is effective in decreasing virus excretion at such moderately high titres, this vaccine need to be further improved eg. in its antigenic content during delivery, formulation with effective oil adjuvants and boosting effect, so as this vaccine can induced high titres in at least 80% of chickens in a single or multiple vaccination.

MATERIALS AND METHODS

Viruses

The Malaysian vaccine virus strain A/duck/Malaysia/8443/04 H5N2 isolated from ducks was developed as an inactivated whole-virus oil emulsion vaccine at Veterinary Research Institute, Ipoh. The pre-activation titre was 10^3 EID_{50}/0.1ml. Cleavage site sequencing and the IVPI showed that the virus is of low pathogenicity. Table 1 showed the comparison between the H5N2 vaccine and H5N1 challenged strain. The challenge virus strain used was A/chicken/Malaysia/5858/04 H5N1.

Compared to the challenge virus, the percentage sequence identity of the HA gene of the vaccine H5N2 and challenge virus H5N1 was 88.2% by nucleotide sequence and 90% by amino acid sequence. As for the comparison of the HAI segment, the nucleotide sequence similarities were 88.3 % and by amino acid sequence was 87.7% (Sharifah et al., 2012).

Table 1: Characterization of the H5N2 vaccine virus strain compared to the challenged H5N1 virus

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Percentage HA gene</th>
<th>Sequence identity HAI segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide sequence</td>
<td>88.2</td>
<td>88.3</td>
</tr>
<tr>
<td>Amino-acid sequence</td>
<td>90</td>
<td>87.7</td>
</tr>
</tbody>
</table>

Vaccination of SPF birds and selection of chickens with high HI titre ≥ 128

Thirty two-week old SPF chickens (raised in Veterinary Research Institute, Ipoh, Malaysia) were vaccinated with 200µl of the H5N2 vaccine via the SQ route. In our studies (Sharifah et al., 2012) we found that 100% of birds had seroconverted by week 4 post vaccination and demonstrated the highest titres at this week of vaccination. At week 4 post vaccination, twelve chickens with high HI antibody titres of 7 log_{2}-9 log_{2} (128 - 512) were selected, tagged and challenged with 200µl H5N1 virus by the intranasal route with 10^{6.0} EID_{50}/0.1ml of the virus. This dose was earlier shown to induce full mortality of unvaccinated controls with a MDT of 36 - 48 hr.

Challenged of the chickens with HPAI H5N1 virus was conducted in a negative pressure isolator cabinet ventilated with HEPA-filtered air in a NATA-certified biosafety level-3 facility of VRI, Ipoh. Water and feed were provided at libitum. Three SPF birds of the same batch and age that had not been vaccinated with the H5N2 vaccine were used as the challenge control. Mean Death Time was determined for all birds that died.

HI assay

The detection of antibodies after vaccination was studied by the HI assay performed according to the WHO manual on Animal Influenza diagnosis and Surveillance (WHO/CDS/CSR/NCS/2002.5). Serum samples were diluted 2 fold, with the initial serum dilution at 1:2. Titres > 3 log_{2} were considered positive. The serological response was evaluated for all birds before and after vaccination. The HI test was performed in V-bottom 96 well microtiter plates with 8 HAU/50µl of homologous inactivatedH5N1 antigen per well.

Oropharyngeal and cloacal shedding of virus

Clinical signs were monitored daily for one week post-challenged. Cloacal and oropharyngeal swabs of each of the chickens were sampled at 3 days post challenge for virus reisolation. The cloacal and oropharyngeal swabs of chickens with similar HI titres (i.e birds with HI titres of 7 log_{2} (128), 8 log_{2} (256) and 9 log_{2} (512) were pooled and virus isolation performed in 9-11 day SPF embryonated eggs using standard procedures (Krauss et al., 2004). Personnel of the Virology Lab of VRI who conducted the
RESULTS

Shedding of challenged virus

Twelve chickens with high HI antibody titres of 7log_29 log_2 (128-512) did not show any clinical signs and survived the challenged. Table 2 showed the HI titre at which the chickens were challenged, the morbidity, mortality and virus re-isolation data. H5N1 virus was however, isolated from the pooled cloacal swabs of the group with HI titre of 128 and 256, but not from the pooled oropharyngeal swabs. No virus was isolated from the pooled samples of the other two groups i.e. the group with HI titre: 256 and HI titre: 512. The H5N1 virus was only detectable at the third passage in SPF eggs and the titre was calculated to be 1 log 10 EID_50/0.1ml. This shows that chicken/s in the HI titre: 128 and 256 group of chickens shed detectable amount of challenge virus by the cloacal route. This is a small study limited by space of the BSL-3 facility, to make statistical inferences of protection associated with these moderately high titres not possible.

DISCUSSION

From our previous study (Sharifah et al., 2012), we showed that with a single vaccination of commercial birds with the inactivated whole H5N2 vaccine that was developed at VRI, Ipoh, the HI antibody titers did not rise to high levels. The highest HI titre achieved was 9 log_2 (512) and only in 4% (1/25) of vaccinated chickens. Titres of 7-9 log_2 (128-512) was only achieved at week 4 post vaccination (Sharifah et al., 2012). In this study, out of the 30 SPF chickens vaccinated only 40% (12/30) achieved a titre 7-9 log_2. From the 30 vaccinated birds, only 5, 4 and 3 chickens attained an HI titre of 128, 256 and 512 respectively, at week 4 post vaccination. In this study, we inoculated 30 SPF birds with the aim of selecting moderately high HI titred chickens for efficacy and shedding evaluation. From the 30 vaccinated birds, only 5, 4 and 3 chickens attained an HI titre of 128, 256 and 512 respectively, at week 4 post vaccination. In this experiment we wanted to determine whether chickens with HI titres of 7-9 log_2 (maximum titres achieved using this vaccine in a single vaccination) can still protect birds against morbidity, mortality and shedding. There were no clinical signs observed in chickens after challenged. However, chickens seemed to excrete virus at 3 days post challenged via the cloacal route. The loads of virus isolated from the cloaca however, was very much reduced (10^2 EID_50/0.1 ml) compared to the load of virus reisolated from the cloaca and also the oropharynx of the challenged control unvaccinated groups. This study confirms that heterologous vaccine and moderately high titred responses protected against clinical signs and mortality, and significantly decreased shedding after intranasal challenge, but they did not fully prevent infection or provide sterile immunity. This inactivated H5N2 vaccine sharing an 88.2 % nucleotide and 90% amino acid similarities with the HA gene and protein respectively of the challenge H5N1 virus, was able to protect chickens with moderately high HI titres of 7 - 9 log_2. Although this vaccine protected chickens with moderately high titred-HI antibody against challenged, chickens with lower HI titres were not protected (Sharifah et al., 2012).

Table 2: Response of 12 SPF chickens (at 6 weeks old) with moderately high HI titres after a single vaccination with the H5N2 inactivated vaccine and challenged with pathogenic H5N1 virus

<table>
<thead>
<tr>
<th>Challenged time</th>
<th>No of birds</th>
<th>HI titre</th>
<th>No. of morbidity</th>
<th>No of mortality</th>
<th>Isolation of H5N1 from pooled samples (titre of virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4 post-vaccination (6 weeks old)</td>
<td>5</td>
<td>128</td>
<td>0/12</td>
<td>0/12</td>
<td>H5N1 virus was reisolated at the third passage in SPF embryonated eggs (EE) only from the pooled cloacal swabs of chickens with HI titre of 128 and 256.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>256</td>
<td></td>
<td></td>
<td>Virus load in the cloacal swabs from chickens of both HI titres was 10^3 EID_50/0.1 ml</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>512</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls 6-wks old SPF chickens</td>
<td>3</td>
<td>&lt; 2</td>
<td>3/3</td>
<td>3/3</td>
<td>H5N1 virus was reisolated from the pooled samples from cloacal swabs and oropharyngeal swabs of dead chickens.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H5N1 virus reisolated at 1st passage in SPF EE. Virus load was 10^3 EID_50/0.1ml</td>
</tr>
</tbody>
</table>

Boostering might result in chickens achieving much higher HI titres, however, this was not done for this vaccine.

All the studies showed that no matter how high the titre achieved by vaccination with a heterologous NA vaccine, high titres still cannot afford sterile immunity to all the birds. Some of the reasons for this could be due to such factors as host genetic and immune system, immunosuppression by other diseases the level of virulence of HPAI strains and partial cross protection, where for example, any one virus that escape the immunity, has the ability to multiply and replicate in the susceptible cells of the oropharynx and the intestines, i.e. areas not reachable by humoral HI antibodies.

In this study we were not able to isolate virus from the oropharynx, however, many workers including Swayne et al. (1999) and Kumar et al. (2007) were able to isolate the challenge virus from the oropharynx. The load of virus in the oropharynx was shown to be higher than in the cloaca. According to Swayne, a 100% homology between the haemagglutinin of vaccine and the challenge virus can protect birds against clinical disease but did not result in the prevention of infection by the challenge virus and shedding from the oropharynx. The differences in total amino acid sequence of the HA1 protein of vaccines, however, was also shown not to correlate with reductions in challenged virus titres shed from the oropharynx or cloaca.

Many workers have reported that vaccination cannot prevent infection whether the vaccine is heterologous or homologous to the virus challenged strain, therefore it is essential that as long as vaccination decreases virus excretion to levels that are insufficient for virus transmission within poultry flocks, than the vaccine should be acceptable. The variable results achieved by the various workers reflects the diverse variables used in each of the studies which include the use of different vaccines, and challenge viruses, different routes of administration of challenge viruses and the different doses and antigenic content of the vaccine at delivery.

Controlling of widespread transmission of the H5N1 virus is a major issue in countries where H5N1 is present and the use of inactivated vaccines is an effective control strategy (Swayne and Suarez, 2000). However, for eradication purposes, a mechanism for the differentiation between infected and vaccinated chickens needs to be introduced (Capua, 2007). It is clear that, because of the human health implications of AI infections, control plans must aim at the elimination of the infection, based on any strategy that is chosen.

REFERENCES

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