Immunopathology in Lungs after Intranasal Challenge with Live Virus in EHV-1 Recovered Murine Model of EHV-1 Infection: Lessons Learned From Unexpected Findings

Keywords: EHV-1; Infection; Horses

Abstract
Equine herpes virus (EHV-1) causes widespread infection among horses worldwide. Virus causes respiratory disease, abortion, neonatal death, paresis, retinopathy, viramea and becomes latent. Horses show transient immunity after EHV-1 infection, where immune responses have been observed to decline after a few months of infection and recovered horses are prone to EHV-1 reinfection. Due to transient immune responses, effective and lasting vaccination to EHV-1 remains a challenge. In an HSV murine model, mice provide solid protection and recovered mice could not be re-infected. In this study we infected mice with EHV-1 intra nasally and after five months, mice were re-infected with EHV-1 along with the previously placebo control. It was expected that mice that had recovered would show some level of protection, but in fact they showed unexpectedly severe clinical signs and more deaths on reinfecion. Reinfected mice showed severe breathing difficulties, abdominal breathing, weight loss and death compared to mice infected for the first time. The answers to the worst clinical signs came from post-mortem and histopathological findings. Lungs of challenged mice showed severe consolidation and profound infiltration of inflammatory cells such that the normal parenchyma and architecture of lungs were completely lost. The results of this study suggest that immunoreactive pathological mechanisms exists and should be considered in designing intranasal vaccine preparation for EHV-1 and possibly for other respiratory infections.

Introduction
Equine herpes virus type 1 is a respiratory infection and is widespread among horses. This virus causes respiratory disease, causes cell associated viraemia and viraemic cells disseminate the virus to all parts of body. The virus crosses the placenta leading to abortion without premonitory clinical signs, and neonatal death follows [1-8]. The virus also disseminates via viraemia to the CNS leading to myeloencephalopathy (paresis) and retinopathy [9,10]. Equine herpes virus is an alpha herpes virus and becomes latent like other alpha herpes viruses and virus can become reactivated [1,11,12]. Virus reactivation during periods of stress causing clinical disease and virus shedding has been reported in horses and after administration of corticosteroids[1,5,13]. This virus is so wide spread and ubiquitous among horses that it is often difficult to obtain a horse free of EHV-1 infection to study the pathogenesis of the virus. A murine model was established to study pathogenesis and immune response to this infection [14,15]. This model since then has been widely used to study the pathogenesis of latency abortion, vaccine efficacy and antiviral efficacy [11,17-19].

Natural immunity to this virus after natural infection in horses or experimental infection in specific pathogen foal is short-lived and horses could be re-infected with the virus within 3-5 months of infection. This is a great challenge to control the spread of this infection in horses due to the transient and short-lived nature of the immunity and as a result development of an effective vaccine remains a challenge [2,20-22].

In herpes virus infection the nature of humoral immune response is very complex and many elements of antibody and cell-mediated immune responses are involved in combating the viral infection. Humoral immune responses are important not only in neutralizing extracellular virus but also in cooperating with non-specific effector cells such as natural killer cells, macrophages and antibody-dependent cell-mediated cytotoxicity to lyse the infected cells [23-27]. Humoral antibodies are relatively ineffective against intracellular virus dissemination which is characteristic of herpesvirus (HSV) infection, and cell mediated immunity responses have been shown to be effective in limiting HSV infectious [28-30]. Although immunity to herpes virus infection is generally more dependent upon the cell-mediated immune responses than humoral responses so far the resistance to the re infection in EHV-1 has only been correlated with the humoral antibody response [31]. An effective immune response requires a synchronization of a number of different physiological and immunological events. Vaccination has been highly successful in control of other viral infections either as an inactivated virus (rabies or Polio) or subunit vaccine (hepatitis B) or yellow fever virus or smallpox vaccine [32-34]. In view of the impressive record of vaccination programs, it seems surprising that attempts to make an effective vaccine against EHV-1 have been very disappointing [22,35-37]. Means to protect the horse from reinfection depends on the antigenic stimulation of antiviral immune response of the horse either with live or inactive vaccine both of which have been tried [1,21]. In either of these studies, only virus isolation was attempted but no attempts were made to study local reaction to lungs on challenge with the virus. Although infections were followed by the appearance of neutralizing and complement fixing antibodies, it appears that the mere presence of these antibodies are not sufficient to guarantee
Intranasal inoculation of mice working stock was stored at -70°C in small volumes till used. The virus was grown at 37°C in a humidified atmosphere containing 5% CO₂. The suspension of virus in Eagles Minimum Essential Medium [EMEM] containing 2% fetal calf serum (FCS) and 1% carboxymethyl cellulose (CMC) at a low multiplicity of infection (m.o.i.) and the working stock was stored at -70°C in small volumes till used.

**Intranasal inoculation of mice**

BALB/c female mice were inoculated intranasally (10⁷ p.f.u. per mouse) and observed daily for evidence and progression of infection. Mice were slightly subjected to light anesthesia with ether and 20 μl in volume of virus suspension was placed in each nostril until all was inspired, which occurred over the course of a few seconds. When all mice had been inoculated, the surplus virus was titrated to confirm the dose administered. Mice were euthanized at various time points and their organs were titrated for virus isolation. Heparinized anticoagulated blood was collected and infectious centre assay for viraemia was performed (see below).

**Reinfection with live virus previously inoculated with live virus**

Twenty-eight BALB c female mice were divided in two groups. Group I was inoculated with live virus (10⁷ p.f.u. /mouse) and groups II was inoculated with an uninfected RK cell lysate. Clinical signs were observed in both the groups after inoculation. Group of mice from the same batch were kept which were not inoculated and would be used as primary infection group five months later along with a placebo group control in a challenge study.

Five months after primary infection both groups were inoculated with the same dose of virus (10⁷ p.f.u. /mouse). Their clinical signs were observed as noted. Four mice were killed on day 3 and three mice on day 5 p.i in both the groups along with three mice in negative control group. Their respiratory organs were removed and processed for virus isolation. Anticoagulated blood was also collected under terminal sedation via cardiac puncture in heparinized plastic vials. Buffoy coat was collected and infectious centre assay for viraemia was performed to compare viraemia in both groups (see below).

**Clinical assessment**

Mice were weighed and examined daily to determine the extent of the infection and progression of the infection and the weigh change of each animal individually. Their clinical signs were noted subjectively. Obvious signs such as ruffled fur, crouching in corners or generalized crouching, dyspnea, abdominal breathing, hunched back, athetoid movements and deaths were recorded.

**Virus isolation from murine tissues**

Groups of mice from each infected group were sacrificed by pentobarbitone sodium injection. The various respiratory organs were minced with scissors and homogenized in an electric blender in 2 ml quantity of Eagles Minimum Essential Medium [EMEM]. The suspension was sonicated for 1 min at 0°C and centrifuged at 3000 rpm for 10 min at 0°C to remove cellular debris. Dilutions of the supernatants were performed in EMEM and sample was inoculated on to confluent RK-13 monolayers. After 45 min of adsorption, EMEM containing 2% fetal calf serum (FCS) and 1% carboxymethyl cellulose (CMC) was added, the monolayer cultures were incubated further and examined after 48 to 72 hours incubation. Monolayer’s were stained with crystal violet and plaques were counted using an inverted Nikon microscope at X6, 12, 25, 50X magnification.

**Infectious center assay**

Blood (2mg/ml EDTA) was collected direct from the heart following the induction of terminal anesthesia. The blood was centrifuged in microfuge tubes for 5 min and the buffy coat was removed by micropipette. The buffy coat was mixed in 0.9 ml of sterilized distilled water for 1 min to lyse the erythrocytes (flash...
llysis). The osmotic balance was restored with sterilized [Phosphate Buffered Saline, PBS] PBS X 10 at 1/10 volume of buffy coat suspension in "whirlimix" and centrifuged for 5 minutes to pellet the buffy coat cells. The cells were counted in a haemocytometer and precise number of leucocytes was then added to the confluent RK-13 monolayer. After 30 min of incubation to allow the cells to settle down onto the monolayers, overlay medium with 5% FCS was added and incubation continued for further 5 days. The development of plaques was determined and number of plaques representing infectious centres per 10^6 cells was counted. One plaque represents one infectious center equivalent. In the absence of cytopathic effect or plaques monolayers were harvested centrifuged at 3K [3,000] rpm for 10 min to pellet the cells. These cell pellets were mixed in virus isolation medium and sonicated in ice cold water in sonic bath for one min and pipetted onto fresh monolayer and incubated for any plaques.

**Postmortem findings**

Postmortem was performed on mice that died of acute infection or mice that were humanely euthanized along with the controls at various time points. Gross observation like the colour and texture of lungs and any other gross abnormalities were noted and recorded. Any gross appearance of lungs including any consolidation, color change or spongy feel of lung was examined, and findings recorded. Small sections of lungs were fixed in 10% formal saline, paraffin embedded, thin sections were cut and subjected to Haematoxylin and Eosin (H and E) staining to evaluate histopathological changes in both the groups.

**Histopathology**

Mice were humanely euthanized by pentobarbitone sodium. Small sections of tissues were carefully excised with a scalpel and fixed in 10% formal saline. Tissues were dehydrated and embedded in automatic processor machine in following sequence. Tissues were dehydrated through ascending alcohol concentration at 50%, 70%, 90% and 100% and followed by three changes in acetone for 2 h and two changes in molten paraffin was made and brought back to room temp. Thin sections (5µm) were cut from at approx. 100 µm intervals. In level sectioning (sections were cut after each 5mm) were also performed. These sections were mounted on glass slides and stained using the standard hematoxylin-eosin (H&E) methods of staining.

**Results**

**Clinical signs after primary intranasal inoculation**

All infected mice began to demonstrate clinical signs of infection 48 hours after inoculation and by day 3 p.i. all animals were hunched with ruffled fur and infected animal appeared smaller in size and began lose weight which was confirmed by measuring the weights of each mouse daily. Infected mice showed continuous weight reduction for 4 to 5 days and then gradually started to recover. From the third day irregularity in breathing was noted which became progressively worse by day 5 post-infection. About half of the animals demonstrated clinical signs of recovery from infection. Mice were also placebo infected with cells lysate only. None of this group showed any loss in weight or any other clinical signs of reaction to the monolayer cell lysate administered (Figure 1).

**Clinical observation after challenge of primary infected mice**

Mice started showing clinical signs of the disease on day 2 p.i. However, on day 3, mice were inoculated with live virus and were given live virus again). Challenged group showed more pronounced clinical signs and difficulty in breathing compared to group which was administered virus for the first time. There was a significant reduction in the weight of mice in this group compared to groups who exposed to same virus for the first time and mice looked smaller in appearance (Figure 1). Placebo inoculated mice in primary infection or placebo control in the challenge study did not demonstrate any clinical signs.
**Virus isolation**

Virus was isolated from respiratory target organs (turbinates, bones, and lungs). No significant difference was noted between the two groups (mice exposed to virus for first time or second time) in the turbinates bones on day 3 p.i but virus titres were significantly lower in challenged group on day 5 post infection. Reduction in the virus titre was found in the lungs on day 3 and on day 5 p.i. in mice that had been inoculated previously with the live virus and this difference was highly significant (p<0.05) and no virus was isolated from challenge group on day 5 p.i (Figure 3, Table 1). This was in contrast to the clinical finding observed where more severe clinical signs like abdominal breathing and deaths were observed in the group of mice that were administered the same strain and dose of live virus five months later compared to groups who has been exposed to the virus for the first time (Figure 1).

**Infectious center assays (IC)**

Anticoagulated blood was collected, and IC assays were performed. Infectious centers were detected in both groups on days 3 and day 5 p.i. The infectious centers were significantly low on day 3 post infection but only two surviving mice were used on day 3 in this group (Table 2). So the level of confidence of significance could not be applied. However on mean values this difference in ICs was comparatively low (8 times lower) to a group of mice previously inoculated with live virus (Table 1). No viraemia was detected in this group on day 5 which was inoculated with virus for the first time but one mouse in challenge groups was positive for viraemia but infectious centers were very low (7 in number) (Table2).

**Table 1:** Table showing infectious centre-forming cells in the blood of mice superinfected with live virus five months after primary infection with live virus.

<table>
<thead>
<tr>
<th>Individual Mouse No.</th>
<th>Time Post Infection (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>409</td>
</tr>
<tr>
<td>2</td>
<td>238</td>
</tr>
<tr>
<td>3</td>
<td>N.D.</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
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<tr>
<td>5</td>
<td>17</td>
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<tr>
<td>6</td>
<td>79</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
</tr>
</tbody>
</table>

a: Mice Were super infected with EHV-1 (10⁷ p.f.u./mouse). In the primary inoculation animals (Mice 1-4) received RK-13 cells while animals (mice-57) received EHV-1 (10⁷ p.f.u./mouse). Both groups were superinfected five months after primary infection. b: These data represent the results obtained for individual mice expressed as the number of infectious centre forming cells/10⁶ leucocytes.

N.D. Not done. Mice died

**Figure 3:** Histogram showing the virus isolation from the respiratory tissues of mice challenged with live virus (10⁷ p.f.u./mouse) five months after primary infection with live virus. Their organs were tested on days 3, and 5 p.i., each bar represents virus titres (geometric means and + S.D.) of four mice process at each time.

**Figure 4:** Photo micrographs howing the histopathological changes after challenged with livevirus (10⁷ p.f.u./mouse five months after previous inoculation with livevirus (107p.f.u./mouse). (a) lung of a mouse showing histopathology at day 3 post challenged previously inoculated with RK – 13 cell lysate and infected with livevirus 107p.f.u./mouse). Note the sloughing and ballooning of the bronchiolar epithelial cells (largearrow). (b) Lung of a mouse 3days after the challenge with livevirus (107p.f.u./mouse). The normal alveolar architecture is completely lost (small arrow) and occasional ballooning and desquamation of epithelial cells lining the bronchiolo can be seen (arrow). A special feature was the peribronchial and peri vascular cuffing of mononuclear cells (curvedarrow) (X193). (c) Similar section at higher magnification (X240) showing in filtration of inflammatory cells (curvedarrow) around the blood vessels. Occasional ballooning along with the desquamation of bronchial epithelial cells (arrow) Infiltration of Inflammatory cells and lymphocytes (dark nuclei) (curved arrow) around the blood vessels, within the epithelial lining of the bronchiolo and in alveolar parenchyma can be seen ( X240).

**Figure 5:** Slide taken from archives showing effect of Spanish flu on the human lung. There is wide spread of lymphocytes and damage is so severe that red blood cells are visible in the parenchyma of the lung with no visible alveoli.
The murine model of human herpes virus provides valuable information with regard to pathogenesis, immune responses, latency, vaccination, and antiviral efficacy [12,24-26,51-52]. The virus also becomes latent in the trigeminal ganglia. Studies in mice show a similar pattern of virus pathogenesis as seen in humans [24, 52-55]. After primary infection there is a solid immune response and mice cannot be reinfected. After primary infection of herpes simplex virus there is a strong immune response and human subjects are not easy to be reinfected with the virus. Similarly, latently infected subjects are protected from a second infection or reinfection from HSV but the virus reactsivate and causes disease [12,56]. This is closely observed in married subjects where one partner reactsivates and may show the signs of the illness but the other partner may not become reinfeected or show any signs of disease perhaps due to primary subclinical infection or low grade primary infection and the presence of immunity hence protection to reinfeciton (unpublished personal observation of medical student couples).

In the present study mice were challenged with the same dose and same strain of virus five months after primary infection. It was expected that these mice will provide some sort of protection after recovery from the live virus infection but challenged mice demonstrated worse and more severe clinical signs of infection compared to the same age group of mice who were inoculated with the same dose of virus for the first time, albeit there was less virus recovery from the challenge group (Figure 3, Table 1). Virus was isolated from turbinate bones and lungs from both the groups, but the difference was not significant in virus titres recovered from turbinate bone on day 3. But it was significant on day 5. Low virus titres were obtained from the lungs on day 3 and 5 p.i. from group of mice that recovered from primary infection after 5 months of primary infection (Figure 3). The low virus titres difference was highly significant obtained from the lungs on day 5 from challenged group but this did not warrant the clinical signs observed (Figure 1). Lungs are highly vascular tissues compared to nasal epithelium and perhaps more immunoreactive mechanisms were involved on challenge with live virus in the highly vascular tissues.

Virus isolation from the respiratory organs and level of viraemia indicates that recovered mice from primary infection after five months did support the virus replication, but the amount of virus recovered did not warrant the severe clinical signs and greater deaths observed in the challenge group. Challenged mice showed severe abdominal breathing (dyspnea), mice looked very unwell with ruffled fur, hunched back in the corners of their cages and about 30% died. Severe clinical signs did not warrant the low virus titre solation and answers to unexpected findings came from postmortem when histopathological studies were performed (Figure 3, Table 1).

On post-mortem examination of mice who were challenged with live virus five months after recovery, there was profound consolidation and, in most cases, the spongy feel of the lungs were completely lost and lung tissues appeared very hyperemic with a liver colouration. More information about the nature of consolidation came from the histopathological observations.

Histopathological findings on lungs of mice that were given the virus reactivated and caused disease [12,56]. This is closely observed in married subjects where one partner reactsivates and may show the signs of the illness but the other partner may not become reinfeected or show any signs of disease perhaps due to primary subclinical infection or low grade primary infection and the presence of immunity hence protection to reinfection (unpublished personal observation of medical student couples).

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signs. Lung parenchyma of challenged mice was full of inflammatory cells. There was profound infiltration of lymphocytes in tertiary and terminal bronchioles and in many cases infiltration of inflammatory cells were so profound that alveolar sacs and alveolar septae were not visible. At many locations near the terminal bronchioles, it was not even possible to diagnose the tissues as lung tissue due to destruction of alveolar septae and infiltration of lymphocytes (Figure 4b & 4c).

We believe that these infiltrating cells may be of the same type to the cells which took part in DTH response as reported before. A cell-mediated response was noted previously after 78 days the last time point tested is further evidenced by the histopathological observation of the local infiltration of the inflammatory cells and peri-bronchiolar cuffing in the infected site of lung tissue [12]. Before the current in vivo study with histopathological evidence, cell-mediated in horses infected with EHV-1 has been mainly restricted to lymphocyte proliferation assays [21, 37, 57-59]. We believe that current observation also provides unequivocal evidence that cellular part of immune response (immune mediated damage) can cause severe damage or even death and was responsible for the clinical symptoms seen in this study. This infiltration and severity of inflammatory cells was very profound and invading cells damaged the fundamental alveolar architecture in the lung parenchyma. To us these inflammatory cells look like lymphocytes (dark blue big nucleus with little cytoplasm, typical morphology of lymphocytes) at the area of primary, secondary, tertiary and terminal bronchioles and alveoli which are area of virus replication (Awan et al. 1990). It is likely that when virus replicate in these alveolar cells, cells express virus specific peptides in terms of MHC-1 and attract virus specific cytotoxic cells. This probably explains why animals that were challenged after recovery demonstrated more severe clinical signs like difficulty in breathing, look very unwell, exhibit abdominal breathing and died (Fig. 1). This reaction looks very much like a hypersensitivity reaction in the lungs. We believe that the clinical signs and destruction of local parenchyma at the site of virus replication are due to immunoreactive cells. When virus replicates in the cells, the cells express virus specific MHC peptide and these cells then become the target of virus specific cytotoxic cells or CD8 cells. It is also reported that the herpesvirus glycoproteins are expressed on the membrane of infected cells and they are also targets for the cellular and humoral immune responses [29,51,53,54,60].

This study provides vital information re challenge study in respiratory pathogens not only in EHV-1 but other respiratory viral diseases. As we know and worried re H1N1 strain of influenza virus which killed 60 to 100 million people world-wide during the pandemic whether it was in cities or towns or villages during the Spanish flu pandemic. In the first wave of 1916-17 pandemic there was no immunity in the community so the virus hit hard but in the second wave of 1917-18 probably it was the immune response to first infection (cytotoxic cells and ADCC killing) which likely mounted a vigorous attack, caused more damage and took more victims. Historical slides taken from archives (Figure 5) show the effect of influenza virus on the human lung and we made similar histopathological observations in our study (Figure 4b, 4c). The figure (Figure 5) does not show typical pathogenesis of influenza virus on the epithelial lining of respiratory cells but in fact it shows severe infiltration of inflammatory cells and damage caused by these cells were so severe that not only alveolar septae were damaged but blood cells could be seen among the infiltrating cells as pathogenesis of influenza virus is rather different from EHV-1 infection (Figure 5). Though, antibiotics could be useful to stop secondary bacterial infection, it is the over excited cellular part of immunity i.e. cytotoxic killer cells or CD8 cells which may be responsible for more deaths and damage [29-30,32,34,61-64]. However, the roles of CD8 cytotoxic cells were not discovered until 1973 so it was not possible to dissect the lungs to check CD8 cells infiltrate in cadaver’s lungs in 1918. We speculate that a similar mechanism in the second wave of the Spanish flu existed in what we observed in respiratory virus challenged infection in a murine model of EHV-1 infection. In the current pandemic of COVID-19 we are also beginning to see a similar trend of greater severity in the current second wave of COVID-19 which affects pneumocytes type II leading to death of these cells and an atypical pneumonia. We also believe that observations we made could be an asset in evaluating the vaccine against Covid 19 as the severity of infection was seen in vaccinated monkeys and such mechanism could be dissected before any damaging effect of vaccine is observed in human subjects.

Many research groups who are working on vaccines for respiratory viruses or respiratory diseases (influenza, RSV or asthma) did the challenge study after intranasal infection and showed protection in their own way. By examining the data presented in conferences, all studies showed weight loss and even death in challenge studies but none could explain this loss of weight as none went any further to investigate the pathological changes seen in challenged groups who lost weight or died. We are fortunate that we went one step further as it was intriguing to find out reasons of severe respiratory signs, abdominal breathing and death. Post mortem findings and histopathological study provided most of the answers and the reason of the clinical signs, weight loss, and death even though fewer viruses were obtained in challenge group.

We believe that cellular part of immunity i.e. cytotoxic cells or CD8 cells and role of immunocomplexes after vaccination (live attenuated, killed, recombinant or mRNA) should be taken into consideration while designing a vaccine as after vaccination both humoral and cellular immunity will become activated [55]. It is the over excited cellular part of immunity along with basophils [via histamine release and cytokine storm] which can cause damage. Over excited cellular part and hence inflammation could be modulated by drugs and in recent COVID-19 cases, anti-inflammatory agents, e.g. dexamethasone, has been found to be effective in reducing the severity of the disease i.e. local inflammation. Non-steroidal drugs of choice could also be of value to control the over expressed profound cellular response as CTL will kill any cells which express foreign epitopes and hence more damage and enable a cascade of inflammation to commence. In order to see the safety of a vaccine, care should be taken that these epitopes do not trigger severe cellular immune responses (CTL, DTH) as this may lead to the destruction of any cell expressing MHC-1 and CD40L on their surface and this may lead to side effects, like fatigue or in worse scenario autoimmune disorders if mimicry to self-molecule is present. Though only respiratory pathogens are discussed in this manuscript, we believe this may applies to other pathogens and vaccines which may trigger a strong cell mediated immune response. It is, therefore, suggested from our findings that cellular immune responses to epitopes is of paramount importance in.
safety and should be considered in designing and marketing any vaccine to avoid any inadvertent immunopathological damage.

Conclusions

Equine herpes virus (EHV-1) causes wide-spread infection among horses worldwide. After a few months of EHV-1 infection, Immune responses have been observed to decline and recovered horses are prone to EHV-1 reinfection. Due to transient Immunity, effective and lasting vaccination to EHV-1 remains a challenge. In an HSV murine model, mice provides solid protection and recovered mice could not be re-infected. In this study infected mice with EHV-1 intranasally and challenged after five months, showed unexpectedly severe clinical signs and more deaths on reinfection. Reinjected mice showed severe breathing difficulties, abdominal breathing, weight loss and death compared to mice infected for the first time. On histopathological studies Lungs of challenged mice showed severe consolidation and profound infiltration of cells such that the normal parenchyma and architecture of lungs were completely lost. The results of this study suggest that immunopathological mechanisms exist and should be considered in designing any intranasal vaccines.

Contributors

This study was performed in department of veterinary medicine university of Cambridge, Dr. Hugh field is life fellow, Queens College and Dr Aftab Awan is life member of Darwin College and Dean of college of Veterinary Medicine, University of Health and Humanities, Tortola, Virgin Islands. Dr Tulp is president of University of Science Arts and Technology, Montserrat and the University of Health and Humanities, Virgin Islands.

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