A Minor Field Study on Avian Metapneumovirus

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1. ABSTRACT

Avian metapneumovirus (aMPV) is an important pathogen that causes respiratory diseases and reproductive failure in various avian species. The disease was first reported in South Africa in the late 1970s. Later, the disease has been reported from all countries around the world, except for Australia, and has caused economic losses in poultry industry worldwide due to the transient drop in eggs and meat production. In 1998 there was an outbreak in Sweden but due to good biosecurity and effective vaccine control program the disease has been under control since then. In South America the industry of egg-laying hens struggles with many respiratory diseases, such as aMPV, Infectious bronchitis, Laryngotracheitis and Newcastle disease. This study is a field project on the Avian metapnuemovirus and took place in the state of São Paulo, Brazil. The study contributed to a bigger research project at the University of Sao Paulo under the department of Preventive Veterinary and Animal Health where the veterinarians are trying to control the problems with respiratory diseases in Brazilian poultry production. The scope of this study was to detect aMPV in egg-laying hens with respiratory disease and reproductive failure and further sequence the viral RNA and compare it to previously found viral strains. This information is important to obtain accurate vaccination-programs for control of the virus. Organ-samples and blood-samples were collected from five different farms in Bastos, a town in the state of São Paulo with extensive egg-production. Reverse transcriptase PCR was performed to detect RNA of aMPV in the organs of the sampled birds. One out of 66 pools of samples was positive. The sequencing of the RNA was unsuccessful and no phylogenetic tree comparing this strain to other known viral strains of aMPV could be designed. The blood-samples were tested with two types of ELISAs, one indirect ELISA and one blocking ELISA, for antibodies against aMPV. Antibodies could be detected in 85 % of the birds with the SVANOVIR-kit; and in 87 % of the birds using the IDEXX-kit. A majority of the birds had antibody-titers corresponding to previous infection. At the farm where the PCR-positive sample was collected, the birds had not seroconverted in spite of reported vaccination against aMPV one month earlier. The result of this study is consistent with previous conclusions from other studies; that RNA of aMPV is difficult to detect and that vaccine prevention of the disease is sometimes unsuccessful.

2. INTRODUCTION

With a growing world population, the demand for good-quality livestock products is extensive and expected to keep increasing. Brazil is today one of the world's most influential economies but still, approximately 25% of the population in Brazil, or about 50 million people live in poverty (ECLAC, 2010). With the increasing possibility for better life-standard in many countries, such as Brazil, the need for efficient food-production is crucial to meet the demands. Meat from poultry serves as an important source of protein and represents almost one-third of meat produced and consumed globally (FAOSTAT, 2009). Brazil's processed food exports are showing the fastest rate of growth among the world´s major food producers. Brazil is the largest exporter of chicken meat since 2004 and is the third producer after the US and China (União Brasilieira de Avicultura, 2012). However, infectious diseases represent a true challenge for the industry and respiratory diseases in chickens and turkeys can cause enormous economic losses for the farmer due to a transient drop in production. Furthermore, diseases also cause animal suffering and may contribute to excessive and inappropriate use of antibiotics. The prevention of infectious respiratory diseases is crucial and research on safe and efficient diagnostic-methods, improvement of vaccines and use of vaccines are central issues. Molecular epidemiology is an important part of this research and allows a better understanding of diseases from a population point of view. This study is a Minor Field Study of the Avian metapneumovirus in the state of São Paulo, Brazil as well as a global literature review focusing on diagnosis and preventive methods of the disease caused by this virus.

2.1 Scope of the study

The scope of this study was to isolate and sequence the partial RNA genome from field strains of aMPV in chicken from Bastos, Brazil. By designing a phylogenetic tree the strains can be compared to other known strains of aMPV in Brazil. Another aim was to find out the serological status with regard to aMPV in the chickens, and relate this to eventual positive samples and vaccination routines within the flock. To determine the serological status, two types of ELISA-tests were used and compared.

3. LITERATURE REVIEW

3.1 Background

3.1.1 History

In the late 1970s an apparently new and severe respiratory infection in turkeys was reported in South Africa (Buys & Du Preez, 1980). In 1989 it was discovered in chickens, also in South Africa (Buys & Du Preez, 1989b). The disease, characterized by sneezing, tracheal rales, swollen infraorbital sinuses and often frothy ocular discharge, was named turkey rhinotracheitis (TRT). This disease had a devastating effect on the turkey industry of South Africa and the industry has not recovered since (Cook, 2009). In 1986 it was discovered in France (Giraud *et al,* 1986) and then in the UK where the causative agent was isolated (McDougall & Cook, 1986) and identified as a pneumovirus (Cavanagh & Barret, 1988). Shortly after its appearance in France and England, aMPV was also reported from other parts of Europe, the Middle and Far East and Latin America and was soon recognized as a major disease threat in both turkeys and chickens all over the world (Cook, 2009). In the US, it was reported for the first time the year of 1996 in turkeys in Colorado and later in Minnesota (Seal, 1998). Serological evidence has suggested that aMPV is widespread throughout the world and Australia is the only region reported free from aMPV (Gough, 2003 and Lauer, 2001).

3.1.2 Classification and definitions

It was the first, and is still, the only avian pneumovirus to have been described and is the type strain of a new genus, *Metapneumovirus* which together with another genus *Pneumovirus* belong to the subfamily *Pneumovirinae* within the family *Paramyxoviridae,* order *Mononegavirales* (Lamb *et al,* 2009). The disease caused by avian pneumovirus has been termed TRT, swollen head syndrome (SHS) and avian rhinotracheitis (ART), then became known as APV, but is now known by the more accurate name, avian metapneumovirus (aMPV) (Cook, 2009).

Other known pathogens within the same subfamily are human respiratory syncytial virus (hRSV), human metapneumovirus, ovine-, bovine-, and caprine respiratory syncytial virus and pneumonia virus of mice (Easton, *et al,* 2004).

3.1.3 Serotypes

There is only one single serotype recognized and four subtypes within that serotype; A, B, C and D. The subtypes can be differentiated by molecular sequencing and with neutralization tests using monoclonal antibodies (Cook & Cavanagh, 2002). The majority of the different subtypes are of the subtypes A and B which were reported in Israel (Banet-Noach *et al*, 2005), Mexico (Decanini *et al*, 1991), Jordan (Roussan *et al*, 2008), Brazil (Dani *et al,* 1999 and D'arce *et al,* 2005), Japan (Tanaka *et al,* 1995) and many European countries (Giraurd *et at* 1986; McDougall & Cook, 1986; Naylor *et al,* 1997; Hafez *et al,* 2000). Subtype A is present in South Africa (Cook, 2009). Isolates from USA belongs to subtype C (Seal, 1998) and are more related to the human metapneumovirus than to other aMPV (Toquin *et al,* 2003 and Yunus *et al,* 2003). The fourth subtype D has been identified once in Muscovy ducks in France (Bäyon-Auboyer *et al* 2000).

3.1.4 AMPV in Brazil

The virus was first isolated in 1995, from broiler breeders flocks (Dani *et al,* 1999). The first reported virus was a subtype A aMPV and all strains isolated and characterized in Brazil up until 2005 belonged to that subtype. In 2007 subtype B was detected in Brazilian commercial flocks (Chacón *et al,* 2007). Subtype A and B are to date the only known subtypes circulating in Brazil. A recent study has suggested that there could be at least two subtype B subpopulations in Brazil (Villarreal *et al* 2009). Both subtype A and B aMPV are present in wild and synanthropic birds in Brazil (Felippe *et al,* 2011). Further surveillance is crucial for knowing which subtypes are currently more prevalent in the field, whether alternation between type A and B occurs, and whether the introduction of a possible new subtype of aMPV occurs. This data is needed to obtain a good and accurate vaccination program in Brazil.

3.1.5 AMPV in Sweden

The disease is a part of the national serological surveillance program in Sweden and if suspected the veterinarians are obligated to report it. In 1998 there was an outbreak of aMPV in parental flocks of meat-producing chicken in southern Sweden. Attempts were made to extinguish the disease but the spread continued within turkey-breeding companies. Samples were also obtained from a large meat-turkey farm in the same area and despite having no contact with infected breeding flocks, most meat-turkey flocks had high antibody-titers for aMPV but showed no clinical symptoms. The virus was detected by RT-PCR but could not be isolated. Because the "stamping out" campaign proved unsuccessful, a vaccination-program was started in Sweden by recommendation from colleagues in other countries with experience from the disease. The parental flocks were vaccinated with a live attenuated vaccine administrated through drinking water at five to eight weeks of age and one inactivated vaccine was given at the age of 18-19 weeks. A year after the outbreaks the company with milder outbreaks stopped vaccinating the parental birds and serological tests have shown that the flocks have remained free of the disease since then. The company that experienced the worst outbreaks still vaccinates all parental flocks. Both companies still vaccinate the grand-parental flocks. The companies with meat-producing turkeys chose not to vaccinate the turkeys because the birds had not shown clinical symptoms. One year later many of these flocks were still seropositive but none showed clinical symptoms (Personal communication with Siamak Zohari, SVA, 2011-11-23).

3.2 Viral structure composition

The virus genome is unsegmented and composed of single-stranded negative sense RNA of approximately 13.4 kilobases. The genome consists of eight genes that encode for viral polypeptides of which two are glycosylated and three are non-structural virus-specified proteins. The viral polypeptide have been identified as nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix protein (M2), a small hydrophobic protein (SH), surface glycoprotein (G), and a viral RNA-dependent RNA polymerase (L) (Gough, 2003). Figure 1 is a schematic drawing of the genome of aMPV. The pneumovirus virion has a lipid envelope derived from the plasma membrane of the host cell into which the attachment (G) glycoprotein, fusion (F) and small hydrophobic (SH) are inserted. The G-protein mediate attachment to the target cell and the F-protein induce fusion between the cell membrane and the virus envelope. The virus

nucleoprotein (N), the phosphoprotein (P) and the viral RNA-dependent RNA polymerase (L) are associated with the RNA genome and important for its replication (Easton, *et al,* 2004)

Figure 1. *A schematic drawing of the order of the aMPV-genome* (Easton, *et al,* 2004*)*

3.3 Susceptibility to chemical and physical agents

The susceptibility of aMPV has been studied using a strain isolated from turkeys in Minnesota. The virus remained viable in temperatures at -70 and -20 degrees for over 26 weeks, 4 degrees for less than 12 weeks, 20 degrees for less than 4 weeks, 37 degrees for 48 hours and 50 degrees for less than 6 hours. The virus kept its activity for 12 cycles of freezing and thawing. It showed resistance to variable pH within the range of 5 to 9 for one hour. Several disinfectants were effective in reducing the viability of the virus, including ethanol, sodium hypochlorite, iodophos, a phenol derivative and quaternary ammonia. The virus showed surprisingly high resistance and could be recovered on cell culture after seven days of drying in room temperature. In general though, the virus is considered to be quite sensitive (Townsend, *et al,* 2000).

3.4 Species susceptible

Turkeys and chickens are the two most important species susceptible to the infection. Serologically the isolates from turkeys and chickens cannot be differentiated but biological differences between isolates from the two species have been reported and it has been suggested that some turkey isolates do not induce respiratory disease in chickens (Cook *et al*, 1993a). Contrary to this, when comparing isolates from chicken and turkeys on genetic level, no species-specific sequence exists for aMPV (Clubbe *et al,* 2009). Serological and molecular tests have showed that aMPV can occur in several other avian species. AMPV can infect ducks (Toquin *et al,* 1999), pheasants, guinea fowl (Gough *et al,* 1988), ostriches, Canada geese wild sparrows, geese, swallows and starlings (Njenga *et al*, 2003).

3.5 Transmission

Transmission of the virus mostly occurs due to direct contact but the rapid spread in many countries is indicating that transmission must occur in other ways as well. Indirect spread through transports, equipment, contaminated water and airborne spread has been suggested. Although the virus can be detected in the reproductive tracts in laying birds, there are no evidence for vertical transmission (Gough, 2003). The importance of transmission through wild birds has also been discussed and might explain the rapid spread of aMPV across the world since the first outbreak in South Africa (Cook & Cavanagh, 2002). The US turkey outbreaks of APV displayed seasonal pattern with approximately 80% of the outbreaks occurring in spring (April to May) and autumn (October to December), suggesting

that environmental factors such as migratory birds may be involved in the spread of virus. AMPV RNA was isolated from nasal turbinates of geese, wild sparrows, starlings and swallow captured in the north central region of the US. The AMPV genes were examined and showed high sequence identity with isolates from turkeys (90-99 %). In addition, infectious aMPV was isolated from wild Canada geese and from sentinel ducks placed in a pond in close proximity with an aMPV infected turkey farm. Infectious aMPV has also been isolated from Muscovy ducks in France. Although there is evidence that aMPV can replicate in wild birds, the role of migratory birds for spread of the virus is still not clear. Canada, which neighbors to Minnesota has not reported serious aMPV outbreaks in spite of the high incidence of disease in Minnesota (Njenga *et al*, 2003). In Brazil, both subtype A and B are present in wild and synanthropic birds. Differences in both nucleotide and amino acid sequence between subtype A of chickens from different origins and subtype A from wild avian species suggests that this subtype probably has adapted to these birds. The detection of aMPV in wild birds is important for the understanding of the epizootology of aMPV in turkeys and chicken and, therefore, for the planning of biosecurity measures and vaccination protocols for poultry farms (Felippe *et al,* 2011).

3.6 Clinical signs, morbidity and mortality

The clinical signs of aMPV infection are unspecific and characterized by respiratory and reproductive symptoms. The same clinical symptoms can be the result from infection with other organisms such as Newcastle virus, Avian influenza virus, Infectious bronchitis and Avian paramyxovirus type 1. Secondary bacterial infections are not uncommon and the characteristic swollen head in SHS is usually caused by a secondary infection with Escherichia Coli (Gough, 2003; SVA, 2012).

3.6.1   Turkeys

AMPV is of great importance in turkeys, where the virus causes a severe respiratory infection. In younger turkeys common symptoms can be sneezing, nasal and eye discharge, conjunctivitis, submandibular edema, swollen infraorbital sinus, snicking and rales. Secondary bacterial infections can dramatically increase the clinical signs. Young adults experience coughing and head shaking. Morbidity can be as high as 100 %, with mortality ranging from 0.4 % to 50 %. The disease is also associated with a reproduction failure, which is of great importance to farmers breeding turkeys. The decrease in egg production can be up to 70 %. The quality of the eggs can be affected with an increased incidence of thin-shelled eggs and egg-peritonitis. The severe coughing resulting from a lower respiratory tract infection can sometimes lead to prolapses of the uterus in breeding turkeys (Gough, 2003 and Cook, 2000).

3.6.2   Chickens

In chickens the role of aMPV as a primary pathogen was until quite recently questioned and infection may not always be associated with clinical signs. In breeders of layers, disease can affect the quality of eggs and cause a range of reproductive tract abnormalities, such as egg peritonitis, folded shell membranes in the oviduct, misshapen eggs and ovary and oviduct regression. For chicken aMPV also plays a role in the complex disease syndrome "Swollen Head Syndrome" (SHS) (Villarreal *et al* 2007). The clinical features of SHS is

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characterized by swelling of the infra- and periorbital sinuses, torticollis, cerebral disorientation, and opistotonus. In figure 2 there is a hen with severe SHS. The morbidity for SHS is often less than 4 %, but respiratory signs are often widespread in the flock. The mortality is low, usually not more than 2 % (Gough, 2003).

Figure 2: *A hen with severe swollen head syndrome. The picture was taken in Bastos, Brazil in September 2011 by Clara Atterby.*

3.7 Diagnosis

The nature of aMPV with its genetic and antigenic variation as well as the difference in clinical features, require adequate and reliable methods for diagnosing infection. There are several methods for diagnosing aMPV. The virus can be isolated or detected using electron microscopy, immunochemical techniques or molecular techniques such as PCR or serologically by demonstrating specific antibody response to the virus (Cook & Cavanagh, 2002).

3.7.1 Isolation of Avian Metapneumovirus

Avian metapneumovirus can be very difficult to isolate and the success rate might be low. Furthermore, for reasons that are not apparent, virus isolation from chickens is more difficult than from turkeys. However, virus isolation is the only method of choice if live virus is required for the research. Ideally the virus is isolated from nasal secretion and tissue scraped from the sinuses of the affected birds. It has also been isolated from trachea, lung and viscera of affected poults. It is important to collect the samples from flocks that have not yet obtained severe clinical signs as the virus may not be present in sinuses and turbinates for more than six days. Secondary bacterial infections can be the cause of the severe clinical signs seen later and this can be the reason for the difficulties in isolating aMPV from chickens suffering from SHS (Gough, 2003 and Cook & Cavanagh, 2002).

For the primary isolation of aMPV from field material from either turkey or chicken flocks, the method of choice is either the use of embryonated eggs inoculated via the yolk sac or chicken or turkey embryo TOC (trachea organ culture). A multiple approach should be applied to the method of isolation since experience has showed that different isolates can require different methods for

isolation. An example of this is the US isolate of aMPV for which the use of TOC has proven inappropriate for primary isolation Once the virus has been isolated using one of the two methods, the egg-fluid can be inoculated onto cell cultures such as chick embryo fibroblasts (CEF), chick embryo liver (CEL), or VERO cultures. A cytopathic effect, characterized by scattered focal areas of cell rounding and syncytial formation, is seen after a few passages (Cook & Cavanagh, 2002).

To confirm the identity of the aMPV electron microscopy or immunochemical methods can be used. When studied under the negative staining electron microscopy the aMPV virus has a highly pleomorphic appearance, the particles are enveloped and the sizes range from 80 nm to 500 nm (Gough, 2003). There are three immunochemical methods that are used for detecting aMPV in turkeys; immunofluorescence (IF), immunoperoxidase (IP) and immunogold staining. For detecting aMPV in chickens IF and IP are being used. The value of these tests under field conditions has not been fully evaluated, nor have any scientific studies been undertaken to compare the sensitivity and specificity of IF and IP (Cook & Cavanagh, 2002).

3.7.2 PCR – Polymerase Chain Reaction and sequencing

Reverse transcription PCR (RT-PCR) is a more rapid and sensitive method for detection of aMPV compared to virus isolation due to its powerful amplification technique (Cook & Cavanagh, 2002).

If the virus is propagated in cell cultures or TOCs (tracheal organ cultures) the virus is detected more easily by PCR. However, due to time limitations in laboratories, this is not always possible. The samples used for PCR can be tissue samples from internal organs such as lungs, ovary, kidney and enteric tract, samples from the upper respiratory tract such as nasal and tracheal tissue or swabs from nasal, buccal, pharyngeal, tracheal or esophageal tissue. In one study, the success rate of detecting aMPV with RT-PCR in tracheal and esophageal swabs was compared to the success rate with extracted RNA from nasal and tracheal tissue. RNA was detected 5 days post-infection in both materials but the tissue contained inhibitory material that needed dissolving which required a higher dilution in water while extracting the RNA. Comparative studies between detecting viral RNA from wet swabs and dried swabs respectively have been performed and showed that both methods are successful. Dried swabs are favored as they can be sent to the laboratory by mail without risking overgrowth of other microorganisms that might destroy the viral RNA (Cook & Cavanagh, 2002).

First-round RT-PCR may be sufficient in detecting aMPV and is sometimes preferred to nested-PCR because it minimize the possibility and consequences of cross-contamination with DNA made during the first PCR. However, first-round PCR sometimes fails to detect viral RNA of aMPV whereas nested PCRs detect it. Even with nested RT-PCR the detection rate can be low (Cook & Cavanagh, 2002). Today, a common approach for detection of aMPV by PCR is to amplify the G-gene of the genome. For detecting aMPV subtype A and B the primers G6- in conjunction with G1+ is being used to prime the RT reaction. The 444 base pair product can be used in a nested PCR where the primer G5- is used along with G8+A for subtype A and G9+B for subtype B, generating cDNA of 268 and 361 base pairs, respectively (Cavanagh et al, 1999).   Another approach to detect

subtype A and B is to amplify the N-gene of the genome using primers Nd/Nx described by Bayon-Auboyer et al. (1999). The primers bind in to a conserved area of the N gene and the RT-PCR produce a 115 base pair fragment. The positive samples can be further nested to determine which subtype it is using the primer Ga in conjunction with G12 for subtype A and G2 for subtype B (Banet-Noach, 2005).

The fusion (F) protein gene of a subtype A virus was the first gene to be cloned and sequenced. Since then, all the genes of the subtype A virus have been sequenced (Cook & Cavanagh, 2002). For subtype B, all genes except for the L gene have been sequenced. Sequencing of all genes from subtype C has also been reported (Govindarajan et al, 2004). For subtype D, the G protein and parts of the F and L proteins, have been sequenced (Bäyon-Auboyer et al, 2000).

Sequencing have revealed moderate to extensive differences between the four currently known subtypes. The F protein of subtype C virus has approximately 72 % amino acid identity with the F protein of subtypes A and B. Between the A and B subtypes the identity of the same gene is 83 % (Seal et al, 2000). For the subtype D, the F gene has been compared to the F genes from subgroup A, B and C and revealed amino acid identities of 70-97 % (Bäyon-Auboyer et al, 2000). There is also diversity within subtypes. A study performed by Naylor et al. (1998) compared the F and G protein of subtype B from viruses isolated for a decade from several different countries. Both protein showed >98% amino acid identity and the conclusion was that very little change had occurred. As mentioned earlier, the great majority of aMPVs detected and sequenced have been subtypes A and B, with the exception of the US where subtype C is dominating. For subtype D, only two isolates have been detected in France.

If subtype-specific RT-PCR is the only method used for detecting aMPV, eventual new subtypes could easily go undetected. Bäyon-Auboyer et al. (1999) suggest that a N gene sequence-based RT-PCR for initial detection of aMPV should be performed, followed by G gene based subtype-specific RT-PCR in positive samples. This was suggested because all the known subtypes up until then (A, B and C) had been detected using RT-PCR targeting the N-gene. Subtype D was identified by sequencing the G-gene but no oligonucleotide primers used to characterize subtype A or B aMPVs were able to amplify the G genes of the isolates. The G-gene was amplified by using primers specific for flanking genes (Bäyon-Auboyer et al, 2000).

Even with the sensitive PCR-method, aMPV can be difficult to detect. This can be due to a poor replication rate of the virus. Some strains of aMPV always have a low rate of replication but factors such as previous vaccination and simultaneous infections can also decrease the replication, making it more difficult to detect with PCR. Previous aMPV-vaccination is expected to reduce replication of the virus. A simultaneous infection with IBV or IBV-vaccine can interfere with the replication on aMPV and therefore decrease the possibility to detect aMPV (Cook & Cavanagh, 2002). It can also be difficult to detect RNA from vaccine. Compared to IBV where RNA from the IBV vaccine can be detected several weeks after vaccination, aMPV RNA vaccine can go completely undetected. (Cavanagh et al, 1999).

3.7.3 Serology

Diagnosis of aMPV infection in both turkeys and chickens are usually done serologically. ELISA is the most common serological way of diagnosing aMPV infection in chicken and turkeys (Cook, 2000). Other serological methods like serum neutralization and indirect immunofluorescence can also be used (Cook & Cavanagh, 2002).

3.7.3.1   ELISA

There are a number of commercial ELISA-kits available, most of them are indirect ELISAs but some of them use the method of blocking/competitive ELISA. In the indirect method, microtitres plates are first coated with aMPV antigen and the test serum is then added, followed by an enzyme-labeled anti-turkey or anti-chicken conjugate. A substrate/chromophore solution is added and the enzyme catalysis of the reaction gives a colored product. AMPV-specific antibodies are detected by the color change seen, the color is quantified using a spectrophotometer and the enzymatic activity is directly proportional to the antibody concentration in the test serum (Cook & Cavanagh, 2002). For blocking ELISA the plates are first coated with an aMPV antigen and the test serum is then added, followed by enzyme labeled monoclonal antibodies (mabs) targeting specific antigen-sites. If the test serum contained aMPV antibodies the binding of mabs to the antigen is blocked. By adding a substrate, the result is visualized. Thus, the higher the eventual antibody-concentration in the test serum, the weaker the signal (Crowther, 2009).

Although the method is used frequently, there are many reported problems with ELISA as a diagnostic method for aMPV. Considerable differences in sensitivity in particular but also specificity of different ELISA-kits have been reported (Cook & Cavanagh, 2002). When the ELISA-kit fails to detect antibodies this can be due to several factors. The type of virus-strain used as antigen in the ELISA has proven to be important. A study performed by Eterradossi *et al.* (1992) showed that ELISA-kits with antigen-isolates originating from the same country as the antibodies were more successful compared to ELISA-kits where the antigen and antibody were from different geographical areas. The same workers also suggest that the choice of inadequate antigen for serological testing could hinder the early diagnosis of aMPV infection (Eterradossi *et al*, 1995). It is important to note that ELISA tests that detect both subtype A and B might only detect subtype C very poorly (Cook *et al* 1999). Information regarding serological methods for diagnosing subtype D is scarce. When comparing the use of indirect ELISA to blocking ELISA the blocking ELISA could be appropriate for testing sera from a variety of avian species because the test does not rely on conjugate from any particular avian species. When deciding what ELISA kit to use, one should consider factors that influence the data; the type and source of vaccine used, the type and source of the sample, the type of field challenge that might be expected and the type of information required from the test. Cook & Cavanagh (2002) comment on this: "This suggestion that different kits are suitable for different purposes, while justified from the results of that study, is clearly undesirable. The objective for performing the test is likely to be to obtain answers to some or all of the aforementioned questions, not to assume knowledge of these answers in advance." The conclusion would be that until better kits are available it is important to be aware of the weaknesses regarding ELISA.

3.8 Methods of controlling the disease

It quickly became apparent that controlling aMPV infections by means of strict hygienic procedures combined with the use of good biosecurity was not adequate. Live attenuated and killed vaccines soon became the most important method of controlling the disease. Due to the size and high complexity of the poultry industry in most areas the disease is impossible to extinguish, although this was achieved in Colorado, USA. The reason for the success in Colorado might have been the relatively small size and isolated location of the outbreak there, combined with England's expertise concerning the epidemiology of the disease (Cook, 2009). Sweden is another area mentioned by Jones (2010) as an example of successful eradication of aMPV, probably due to the small chicken population in the country combined with careful monitoring and strict attention to biosecurity. The maternal immunity in turkeys fails to protect young turkey poults from infection (Naylor *et al*, 1997b).

3.8.1   Vaccination

For turkeys, effective vaccines were developed and were quickly put into production and became widely used. When administered correctly they provide excellent protection for turkeys of all ages and are being used for meat turkeys as well as layers and breeders. The live attenuated and inactivated vaccines have been shown to stimulate both systemic immunity and local immunity in the respiratory tract (Gough, 2003). Vaccination is usually achieved by spray or ideally by eye drop, but in the future, the *in ovo* route might prove to be even better (Hess *et al,* 2004). A single vaccination may be sufficient to protect meat turkeys throughout their life but reinfection can occur later in life and meat turkeys that are reared beyond 10 to 12 weeks are sometimes revaccinated. A typical vaccination program for aMPV in turkeys would be the application of a live subtype A or B strain combined at day-old poults using a coarse spray, repeated at 7 to 10 days and again at 4 to 6 weeks. The strategy is to produce cell-mediated immunity in the respiratory tract. Breeding stock would additionally receive an inactivated vaccine at 16 to 20 weeks (Gough, 2003).

However, failure of the vaccine is not uncommon and can be due to several factors such as over attenuation of the virus strains leading to a weak immune response, under attenuation leading to severe reaction or poor vaccine administration (Cook, 2009). There are many reports of post vaccinal disease and one underlying reason could be instability of the vaccine (Catelli *et al*, 2006). In experimental conditions, disease has been seen after 4-10 back passages of vaccine in naive turkeys (Naylor & Jones, 1994). In a field study from a turkey farm in Italy it was shown that respiratory disease was caused by a virus originating from a live subtype A vaccine. Particularly interesting was the fact that this flock had not received vaccine for subtype A indicating that aMPV vaccines are able to circulate in the environment for longer than was previously envisaged (Riccizzi *et al*, 2009). Failure of the vaccine could be due to the involvement of several or unexpected subtypes. Though there is evidence for excellent cross protection between A and B subtypes vaccine (Eterradossi, 1995) as well as cross protection to subtype C with both A and B subtypes vaccines in turkeys (Cook *et al*, 1999) there are also studies that in contradiction to this

confirms limited cross-protection between subtypes (Riccizzi *et al*, 2009; Van de Zande *et al,* 2000). Another aspect that has been considered as a factor regarding poor vaccine-results is the interference between other pathogens and the vaccine. A field study performed in Brazil by Cardoso *et al.* (2009) investigated the possibility of low aMPV vaccine performance due to interference by a persistent infection with turkey astrovirus and concluded that this might be possible. Yet another factor could be the ability of the virus to mutate quickly and the possibility that this might allow the virus to avoid an immune response induced by vaccination. This was examined by Catelli *et al.* (2009) where turkey-poults were experimentally infected with the more recent subtype B Italian isolates after vaccination with the common subtype B vaccine, which has the earlier virus sequence. The result of the study was that a majority of the vaccinated birds who were challenged with the "new" subtype B isolate showed severe clinical symptoms and the conclusion was that the field virus had changed in key antigenic regions in order to thrive within a group of well vaccinated birds. Despite these possible problems, vaccination continues to be seen as highly beneficial, provided careful attention is paid to the method of administration (Cook, 2009).

For chickens, the difficulties involving the diagnosis of aMPV, have led to doubts on whether the vaccines are in fact effective. For broilers in particular there are many reports on the failure to control disease for aMPV-vaccines. According to Cook (2009) good diagnosis is essential in order to evaluate the efficacy of the vaccine since aMPV vaccines will not improve the situation in a flock where aMPV is not present.

It has been hypothesized that aMPV vaccines will be more efficacious in the species from which the progenitor virus was derived because of antigenical differences; however protection studies have proven difficult. A study performed by Clubbe *et al.* (2009) compared chicken and turkey derived aMPV subtype B at their underlying genetic level to identify if species-specific regions exist. While numerous sequence differences between viruses were identified, none was specific for the host species. However, because of the possibility that isolates from chickens may replicate more efficiently in that species, the perceived advantage of using chicken-origin strains led to development of vaccines incorporating strains of aMPV isolated from chickens for use in that species (Cook, 2009).

Further, there is a risk of interference between aMPV and IB- or NDV-vaccines since these viruses target the same cells in the respiratory tract. It is therefore advisable to consider leaving an interval of approximately one week between vaccination against IB and ND and administration of aMPV vaccine (Cook, 2009).

3.8.2 *Protection of egg-laying and breeding birds*

For egg-laying birds, in addition to the live attenuated vaccines, effective inactivated aMPV vaccines are widely used. It has been shown both experimentally and under field conditions that the use of inactivated vaccines can provide good protection against the effect of aMPV challenge on egg production and egg quality, although some clinical signs may be seen for a short time after challenge. Therefore, in some countries where it is difficult to license live-attenuated aMPV vaccine, control programs including inactivated vaccines are

being used (Cook, 2009). In a study performed by Villarreal *et al. (*2009) in an aMPV-endemic region of Brazil, comparison was made between three flocks with egg-laying hens that had received aMPV vaccines with two control flocks, also with egg-laying hens, that had not received vaccines for aMPV. Both groups had received vaccination against IBV, NDV and EDSV. Control flocks showed characteristic symptoms of aMPV whereas the vaccinated flocks showed no clinical symptoms.

3.8.3 Biosecurity

Good biosecurity is crucial for commercial poultry and egg production and the severity of aMPV infection is significantly depending on management factors. In terms of biosecurity there are many factors to consider, such as high stocking density, insufficient ventilation and temperature control, poor litter quality, general hygiene, multi-age stock and the presence of secondary pathogens (Gough, 2003). Biosecurity in poultry production is very important but complex and will not be further discussed in this literature review.

4 MATERIALS AND METHOD

4.4 Site of sample collection

Sample-collection took place in Bastos, a town in the state of São Paulo, Brazil. See figure 3.

Figure 3:

Map of Brazil
www.sao-paulo.world-guides.com

Map of the state of Sao Paulo
www.mapzones.com.
Bastos is situated near the star on the map.

Bastos is well-known in Brazil as one of the most important regions for poultry-production. The town is small with about 20.000 inhabitants but the number of birds is great with 15 million layers of hens, as well as four million quails. There are approximately 200 different poultry-farms in Bastos and the farms are located very near each other which in addition to the very low biosecurity are the reason to why the town of Bastos almost can be considered one extremely large flock in terms of disease-control. Given the very high avian population density in the area it is not hard to understand that the infectious diseases are likely to spread easily and infect and re-infect flocks repeatedly. Some of the circulating pathogens in Bastos include Infectious Bronchitis virus, Mycoplasma spp, Laryngiotracheitis, Avian metapneumovirus and Newcastle virus. The routine for disease-control is mainly vaccines, although this strategy has shown varied results. Biosecurity is as compared to high international standards very poor. Figure 4 is a photograph demonstrating the poor biosecurity in Bastos. For most farms there are no routines for visitors in terms of change of clothes and shoes, no restriction for inter-farms visiting per day, very high population density, no physical barriers (i.e. walls) between the flocks and wild birds with highly dirty facilities that enables airborne pathogens to easily transmit from one flock/farm to another. Respiratory diseases are very common and decrease in production is often a symptom to such problems: respiratory diseases are often associated with huge economical loss to the farmer. Though, instead of improving biosecurity and vaccine-routines to

better control the disease-situation the farmers are applying a buffer-method with high quantity of birds, where some flocks are sick while others are healthy and well-producing. The situation in Bastos makes the area well-suited for epidemiological virology studies and extensive research regarding many infectious diseases in Bastos are ongoing at the Department of Preventive Veterinary Medicine and Animal Health College of Veterinary Medicine, University of Sao Paulo (Own observations and interview with Laura Villarreal and Prof. Paulo Eduardo Brandão).

Figure 4. *A typical farm in Bastos, Brazil. The houses are designed without walls and the flocks are situated near each other and near outside vegetation and wild birds. The picture was taken in Bastos, Brazil in September 2011 by Clara Atterby.*

4.5 Sample collection

For three days in September, blood and organs samples were collected from commercial layer hens in Bastos. The farms and flocks were selected based on several factors; owners willingness, presence of respiratory disease, other practical issues and most importantly previously performed epidemiological studies at the chosen site. Our results were used as complementary information to a larger scale research on epidemiological studies on, amongst other viruses, aMPV in chickens and quails in Bastos. Blood-samples were obtained in 14 flocks within five farms and from 13 of these flocks within four farms organ-samples were also obtained. The amount of birds from which blood was sampled ranged from five to twenty per flock with a total of 182 samples. The amount of blood-samples collected were not pre-planned in detail and depended on accessibility to collection-material, willingness of the owner and other practical factors. About three milliliters of blood was collected from the vein on the inside of the wing, vena cutanea ulnaris, and then transferred to a new tube. The blood was stored cool and serum was separated from the blood within 24 hours by centrifugation. Out of the 182 samples, 159 could be used for analyzing, the others were discarded due to small volume resulting in inability to obtain sufficient amount of sera. The amount of birds from which organ-samples (trachea, lungs, kidneys, reproductive tract and enteric content from mostly caecum) were obtained was five per flock, with the exception of one flock where six birds were collected resulting in a total of 66 birds. The samples were pooled specifically for each organ for every flock resulting in pools of five (six in one) birds per pool and five

pools per flock resulting in 65 pools of samples. The amount of birds per pool is standard for this kind of research at USP. The birds were euthanized by dislocation of the neck and afterwards dissected either immediately at site or within three hours at the local veterinary clinic. The organs were stored frozen. See figure 5 for photographs from the sample-collection. The birds chosen for blood-samples and organ-samples showed varied degree of respiratory symptoms where some birds showed none but most showed some respiratory symptoms and several had severe symptoms with nasal and eye discharge and dyspnea. At least one of the birds had developed "swollen head syndrome" with heavy swelling of the infra- and periorbital sinuses. For detecting aMPV it is optimal to collect samples from birds that have not yet started to show severe clinical symptoms, ideally a neighboring flock to one with clinical symptoms. However, it was difficult to explain this in a convincing way to the present veterinarians and owners and therefore most of the birds that were sampled had already developed clinical symptoms. A few birds showed pathological findings when dissected like liverlipidoses, pneumonia, ovarian cysts and hepatosis. Due to practical difficulties in the field it was unfortunately not possible to obtain reliable data for the presence of clinical symptoms or pathological findings and relate this to the samples and result. All of the farms were asked to send a copy of their vaccination-program but unfortunately only two did. According to the vaccination-program, one of the two farms vaccinated the birds for aMPV by ocular administration (PNEUMO RTV 8544 from Intervet) at the age of 14 and 71 days. The birds were also vaccinated for Infectious laryngotracheitis, Bursal disease, Infectious bronchitis, Mycoplasma gallisepticum, Avian Encephalomyelitis, fowl pox, Infectious coryza and Salmonella enteritidis. The other farm did not vaccinate for aMPV but vaccinated for Infectious laryngotracheitis, Bursal disease, Infectious bronchitis, Mycoplasma gallisepticum, Infectious coryza and Newcastle disease. The third farm did not send their vaccination-program but the farm-veterinarian stated that they do not vaccinate for aMPV. The vaccination-program for the other two farms was unknown. The birds were of different breeds; Hisex, Isa Brown, Dekalb brown and Bovan. From two farms, the breeds of the birds were unknown. The age of the birds ranged from approximately 18 weeks to 77 weeks. For three of the farms, the age is unknown. All of the birds were housed in cages, the number of birds per cage was 2-8. The exact size of the cages was unknown but varied amongst flocks and farms. All of the bird-houses had low biosecurity and were designed without doors, most houses also lacked walls. The flocks were situated very near each other, the closest distance observed was six meters.

Figure 5:

Blood-sample from the vena cutanea ulnaris of a hen in Bastos, Brazil.

Clara Atterby dissecting a hen at a farm in Bastos, Brazil.

Both pictures were taken in September 2011 by Caroline Olofsson.

4.6 Laboratory techniques

4.6.1 RNA-extraction and PCR

Pools were prepared by mechanical decomposition of the organs and 1:2 diluted (v/v) with diethyl pyro carbonate (DEPC)-treated water and submitted to three freeze-thaw cycles and clarified by centrifugation at 5000 x g for 15 minutes at 4°C. For every five samples, one negative control was prepared and for each test run subtype A aMPV vaccine was used as the positive control. The total RNA of field and vaccine samples and negative controls was extracted with TRIzol reagent (Invitrogen^{TM}) according to manufacturer's instructions. 3.5 μl of total extracted RNA were denatured at 94°C for 5 minutes and added to the reverse transcription mix containing 1xFirst Strand Buffer, 1mM dNTP, 10mM DTT (dithiothreitol), DEPC-water, 2,5ng random primer and 100 U of the enzyme M-MLV (Maloney murine leukemic virus) reverse transcriptase (Invitrogen^{TM}) to a final reaction volume of 6,5 μl. Reverse transcription was carried out at 37°C for 60 minutes, followed by 10 minutes at 72°C. The reaction creates cDNA from all the available RNA templates. 2,5 μl of cDNA per sample were amplified by PCR in a mix containing 10 x PCR Buffer (Invitrogen^{TM}), 0,625 U Taq DNA polymerase (Invitrogen^{TM}), dNTP, 1,5 mM magnesium chloride, DEPC-water and 0,5 μM of each primer (G6- and G1+) to a final reaction volume of 25 μl. The conditions for DNA amplification were 94°C/3 min for initial denaturation, followed by 30 cycles of 94°C/1 min, 50°C/1.5 min and 72°C/2 min and a final extension at 72°C/10 min. The nested step was performed by adding 2.5 μl of the first round PCR product to a similar mix containing other primers (G5-, G8+A and G9+B) to a final reaction volume of 25 μl. Primers and reaction conditions used was described by Cavanagh et al. (1999) and Juhasz and Easton (1994), to amplify 268 base pair (bp) or 361 bp fragments corresponding to subtypes A and B, respectively, of the aMPV G-gene. Specific rooms with restricted equipment were used for each step (sample preparation and RNA extraction, reverse transcriptase, and first-round amplifications and second-round amplifications followed by electrophoresis). Next, 10 μl nested-PCR product were visualized

after electrophoresis in a 1.5 % agarose gel that was stained with ethidium bromide (0,5 µl/ml). The nested PCR product from the positive sample (268 bp) corresponding to the G glycoprotein from aMPV, a negative control (DEPC-water) and the vaccine sample was amplified to a total volume of 45 µl. The total volume was applied to a slow electrophoresis in a 1.5 % thick agarose gel which was stained with ethidium bromide (0,5 µl/ml).

4.7 Sequencing and phylogenetic analysis

Using a clean scalpel and wavelength (365 nm) ultraviolet light and minimal exposure time, the band containing the PCR product sample of interest was cut out and put into a DNAse-free micro centrifuge tube. The sample was purified using the GFX PCR DNA and Gel Band Purification Kit (from GE Healthcare). First 800 µl Capture buffer type 3 was added to the sample to denature proteins and dissolve agarose. The mix was then applied to the GFX Micro Spin Column and Collection tube where DNA can bind to the membrane. The tube was centrifuged and the flow was discarded. After this, two steps of washing and drying was performed by adding 500 µl Wash buffer type 1 to the GFX Micro Spin column, then spin the tube and discard the collection tube to remove salts and other contaminants from the membrane bound DNA. The GFX Micro Spin column was transferred to a fresh DNase-free 1.5 ml micro centrifuge tube. The last step was to elute the purified sample from the column with 50 µl Elution buffer type 6 (nuclease free water). The tube was spinned to recover the purified DNA, the column was removed and the Collection tube was stored frozen at -20 °C. The RT-PCR products were sequenced in both the forward and reverse direction. First, the purification products was added to a mix and then submitted to another thermocykle-program; 35 cycles of 96ºC/30 sec, 50ºC/15 sec and 60ºC/4 min, with a ramp of 0.7ºC/seg between each temperature. The mix contained 4 µl Big Dye 301, 4 µl Segmented buffer, 0,4 µl primer, G1+ for forward and G5- for reverse, and 11,6 µl sample to a final reaction of 20 µl. DNA sequencing was performed using ABI 3500 to confirm the specificity of the amplicon. All possible sizes of fragments were marked with a nucleotide-specific fluorescent light and detected using a laser.

A phylogenetic tree could not be constructed because the DNA sequencing was unsuccessful. The purpose of the tree would have been to evaluate the relatedness between the detected viral strains to other known strains of aMPV. The sequence was analyzed using a program called Mega 5 that design a phylogenetic tree based on sequence similarity to all other DNA-sequences within a large global database.

Figure 6: *Professor Paulo Eduardo Brandão is supervising at the lab of University of Sao Paulo, department of Preventive Veterinary and Animal Health*

4.8 ELISA-test

For detection of eventual antibodies in collected blood from 159 birds, the serum was analyzed at the lab using the SVANOVIR Avian Pneumovirus - Ab ELISA kit. 62 samples of the same serum were also sent to another lab where the IDEXX Avian Pneumovirus – Ab ELISA kit was used. The results of the tests were then compared.

The SVANOVIR kit procedure is based on the Blocking-ELISA (Blocking Enzyme Linked Immunosorbent Assay). The samples were added to a microtitre plate coated with aMPV antigen. If aMPV-antibodies are present they will bind to the antigen in the well and block these antigen sites. If there are no aMPV-antibodies present, these sites will remain free. Horseradish peroxidase (HRP) conjugated monoclonal antibodies directed to aMPV are added and they will bind to the sites if they are available. In that case, when the substrate is added, a blue color develops marking that the sample is negative. If the sites are occupied by aMPV-antibodies from the sample, the conjugate are unable to bind and will be washed away before the substrate is added resulting in no color which means that the sample is positive. The result was clear and visible to the eye but a microplate photometer was used to measure the optical density (OD) at 450 nm. OD readings from the samples were used to calculate the percent inhibition (PI) by this mathematical formula: (OD Neg control – OD positive control/sample)/OD Neg control x 100. For positive result the PI is <40, for negative result the PI is >30, samples with a PI of 30-40 are considered doubtful.

IDEXX-kit – 62 selected samples were sent to another lab specialized in analyzing blood-samples with the Idexx-kit. The reason for not sending all samples was the unexpected high cost. The choice was made to include serum from all collected flocks. Samples that were considered doubtful on the Svanovir-kit were selective chosen. The IDEXX-kit is based on an indirect ELISA and can, according to the manufacturer also analyze the quantitative titer of the antibodies.

5   RESULTS

5.1 AMPV detection by RT-PCR

One out of 65 organ pools was positive for aMPV. The positive sample was aMPV subtype A and the RT-nested PCR technique amplified the 268 bp fragment of the G-gene (Figure 7). The positive sample was found in flock 42 in pools of trachea. This flock was approximately 18 weeks old and had been vaccinated against aMPV one month prior to the sampling. In the other 65 pools of organs, no aMPV could be detected and amplified with RT-PCR.
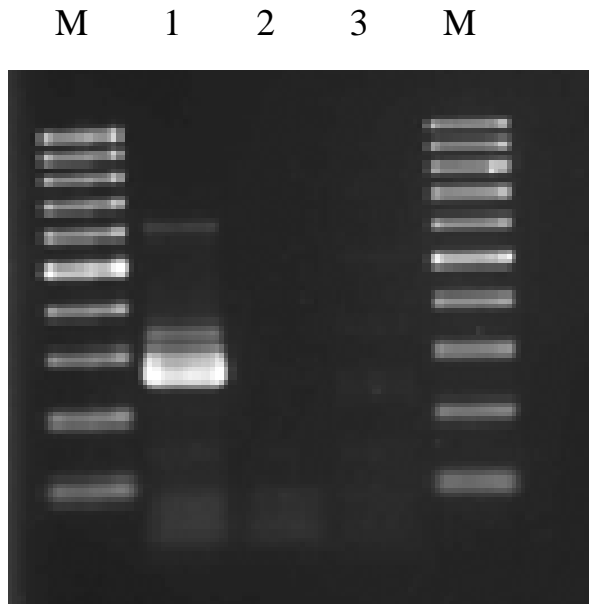
M   1   2   3   M

Figure 7. *RT-PCR of Brazilian strain of aMPV. Lane 1 subtype A field strain; lane 2, negative control; lane 3 subtype A vaccine strain, M, molecular size marker (100 bp ladder) Picture taken by Clara Atterby, 2011-10-14 at University of Sao Paulo, lab of VPS.*

5.2 Sequencing and phylogenetic analysis

The sequencing was unsuccessful and could not be used for phylogenetic analysis.

5.3 AMPV-antibodies detection by two different ELISA-kits

SVANOVIR Avian Pneumovirus – Ab ELISA kit; 135 out of 159 (85%) samples of sera were positive for aMPV, 18 out of 159 (11%) were negative and 6 out of 159 (4%) were considered doubtful.

IDEXX Avian Pneumovirus Ab ELISA kit; 59 out of 62 (87 %) samples of sera were positive for aMPV, while 3 of 62 (13 %) were negative. Since the purpose was to include samples for analyze with the IDEXX-kit that were considered doubtful on the SVANOVIR-kit the samples were not randomly selected. Therefore it would be inappropriate to compare the percentage of positive and negative results between the two tests, although it is obvious that the results are similar. However, on the individual level, the results of the two tests were compared and all samples that were positive with the SVANOVIR-kit were also

positive with the IDEXX, and the same applies to the negative results. Four samples were considered doubtful on the SVANOVIR-kit; two of these were positive and two were negative with the IDEXX-kit.

According to the manufacturer, the IDEXX-kit is able to differentiate antibody-titers corresponding to vaccination from titers corresponding to previous infection. The base-line is derived from serological control of vaccination-programs in Brazil but is not specific for Bastos and the base-lines must be considered to be quite general. 78-91 % of the positive serum-samples had titers corresponding to previous infection, the remaining had titers corresponding to vaccination. It is not possible to achieve a more precise number because of the lack of information regarding the age of the sampled birds (e.g. the number of vaccine-doses received), which influences the titer corresponding to vaccination.

6 DISCUSSION

One out of 66 organ-samples was positive for aMPV with RT-PCR. The occurrence of aMPV in Bastos is however higher than what this result indicates. The results from the ELISA-test show that aMPV is in fact circulating/or has been circulating in the sampled flocks. Antibodies were found in the majority of the birds, also in flocks that had not been vaccinated. It is very difficult to detect aMPV, especially in older birds that have developed an immunoresponse after previous exposure to the virus. Factors that might explain the low rate of detected aMPV could be either suboptimal population for sampling or choice of method at the laboratory regarding PCR-type and primers.

The farms that were sampled all had several flocks with clinical respiratory symptoms. AMPV is a probable pathogen causing disease in these flocks but the symptoms could also be due to other respiratory diseases such as IB, NDV, ILT and Mycoplasma. As described earlier in this paper, for PCR, it is favorable to collect samples from birds that have not yet displayed clinical symptoms since aMPV is replicating most rapidly in the phase of incubation (Gough, 2003). The positive sample was in fact collected from the one flock in the farm that was without clinical symptoms. The challenge in choosing the right flock to collect samples from is to convince the owner that it is more profitable to collect from the flock without clinical symptoms than the neighboring sick flock. To make the owner more willing, samples could be collected using swabs instead of organs and thereby without killing the birds.

The sampled material was handled correctly under the given circumstances and stored frozen throughout the whole process from the time of sampling to the lab. Many samples were positive for other viruses such as IBV and Herpesvirus from which the same RNA-extraction was used and therefore it can be assumed that the RNA-extraction product was correct. The reverse transcriptase-PCR reaction was used which is a good qualitative method for sequencing RNA-viral strains. However, the real time-PCR is a quantitative method that is more sensitive in detecting positive samples and therefore more useful in studies of prevalence. The desired part of the genome for sequencing was the G-gene by using primers specified to amplify the gene from subtypes A and B. This is a common approach for sequencing aMPV in Brazil where subtype A and B are present. Neither subtype C nor subtype D will be detected with this method and in the future it is advisable to use a broader technique, a so-called pan-aMPV RT-PCR assay, while screening for aMPV or else newly introduced subtypes could go undetected. Bäyon-Auboyer *et al.* (1999) suggest that an N gene sequence-based RT-PCR for initial detection of aMPV should be performed, followed by a G gene based subtype-specific RT-PCR in positive samples. This was suggested because all of the known subtypes up until then (A, B and C) had been detected using RT-PCR targeting the N-gene. Subtype D was identified by sequencing the G-gene by using primers specific for flanking genes, no primers used to characterize subtype A or B aMPVs were able to amplify the G genes of the isolates (Bäyon-Auboyer *et al,* 2000).

As can be seen in figure 7, p.25, where the electrophoresis of the positive sample is displayed, the positive control is very faint or even invisible. The positive control failed to work several times during the laboratory work and this is highly undesirable. In this scenario it is impossible to know if the assay per se did not

work, or that the sample contained aMPV RNA. The positive sample was amplified again by another researcher and this time the positive control worked.

The DNA-sequencing was unfortunately unsuccessful. According to supervising Professor Paulo E Brandão the most common reason for this would be an insufficient amount of amplified cDNA. Attempts to repeat this will be made.

The comparison between the two ELISA-kits was not made to a full extent since only 62 out of 158 samples were tested using the indirect method of ELISA (IDEXX-kit). The SVANOVIR-kit can detect aMPV subtype A and B while the IDEXX-kit can detect subtype A, B and C and theoretically, a way to detect subtype C could be through comparison between the two tests (Personal communication Siamak Zohari, SVA, 2012-02-20). However, the results on all tested samples corresponded fully on both tests. Traditionally, the indirect ELISA is the method of choice for aMPV but blocking ELISAs are also common nowadays. The sensitivity is generally higher for indirect ELISA compared to blocking ELISA but the SVANOVIR-kit has according to the manufacturer 98% sensitivity in comparison with other ELISAs. To distinguish antibody-titer corresponding to vaccination from titer corresponding to infection, the PI-value from blocking ELISA can be used but the indirect ELISA is generally more precise in determine the titer and would therefore be recommended for serological control of flocks after vaccination. The antibody-titers results from the Idexx-kit were in this study compared to a general base-line in Brazil. The results would have been more certain if each sampled flock had had a base-line derived from serological control of the vaccination-titer.

The role for aMPV as a primary pathogen in chickens was recently fully established. Gough (2003) writes that aMPV is a very important primary pathogen for turkeys but not for chicken. For chicken, aMPV was established as one of the pathogens in the multi-systemic "swollen head syndrome". Nowadays it is widely accepted that aMPV can cause respiratory and reproductive disease as well as a production decrease in chicken also when the infection is not complicated by other pathogens. However, it appears that this new approach within the world of research is far from established out on the field. A field-veterinarian in Bastos working with chicken stated that he did not believe that aMPV was a primary pathogen for chicken and therefore he did not recommend his clients to vaccinate the flocks for aMPV.

The idea that aMPV is not a field-problem could be due to the fact that aMPV-infection is not always associated with disease, and also because of the difficulties involved in the diagnosis aMPV as the causative agent. It is, as described in more detail earlier in this paper, hard to isolate and/or detect the virus with PCR, especially when the samples are obtained from birds which already have developed clinical symptoms. It is not hard to understand that farmers (and veterinarians) doubt the fact that aMPV causes disease in chicken when the virus is not detected in sick birds.

There are reports of vaccine breaks and vaccine strains evolving into viral-strains for aMPV which probably contribute to the farmers´ apprehension concerning vaccination against the disease. In this study the flock with the positive sample had not seroconverted according to both tested ELISA-kits despite the fact that the

flock had been vaccinated against aMPV one month prior to the time of sampling. The birds were 18 weeks old and had received one dose of vaccine. In one month the birds should have seroconverted. The lack of immune-response could be due to incorrect administration or malfunctioning vaccine.

At some farms from which sample-collection was performed, the farm-veterinarian was asked about the use of antibiotics. The veterinarians were not keen on discussing the subject but one of them said that the birds are treated with antibiotics when they experience respiratory symptoms but the veterinarian could not (or would not) give more detailed information. When asked which antibiotics they used he said enrofloxacin amongst others.

7. CONCLUSIONS

The results of this study are consistent with previous conclusion from other studies; that RNA of aMPV is difficult to detect and that vaccine prevention of the disease is sometimes unsuccessful. Further surveillance is crucial for knowing which subtypes are currently more prevalent in the field, whether alternation between type A and B occurs, and whether the introduction of a possible new subtype of aMPV occurs. This data is needed to obtain a good and accurate vaccination program in Brazil. There was no noticeable difference in the results of the two tested ELISA-kits. Further evaluation and development of serological tests are needed since correct diagnostic methods are crucial in controlling the disease. Regarding the use of antibiotics; if the birds are treated with antibiotics every time they are sick one could assume that large quantities of antibiotics are being used within the Brazilian egg-laying industry and that attempts to treat viral-infections with antibiotics are common. Further research within the area of antibiotic-use in Brazilian poultry would be interesting and also very important.

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