EVIDENCE FOR MAREK'S DISEASE IN TURKEYS IN GERMANY: DETECTION OF MDV-1 USING THE POLYMERASE CHAIN REACTION

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Summary. – Since 1994 tumorous lesions have been monitored in turkeys on three farms in Germany. On one of these farms, chickens also had tumorous lesions. Affected turkeys were retarded in growth, apathic, pale and almost unable to move. The older the animals got, the more indistinct the clinical signs became. Mortality started at an age of 5 weeks and reached between 20% and 60% by the end of the fattening period of about 20 weeks.The aetiological differential diagnosis includes reticuloendotheliosis (RE), lymphoproliferative disease (LPD), lymphoid leukosis (LL) and Marek's disease (MD). Repeated serological examinations did not establish the presence of antibodies against REV, LLV or MDV-1. Cloacal swabs were negative for LL P27 antigen in ELISA. Solid tumors of various sizes as well as diffuse infiltrations were predominantly seen in the liver, spleen and kidney. Pleomorphic cell infiltration was rarely noted in the *plexus brachialis* and *nervus ischiaticus*. Herpesvirus of turkeys (HVT) was the only virus isolated from buffy coat cells derived from affected turkeys in chicken embryo kidney cell (CEK) and chicken embryo fibroblast (CEF) cultures. Use of polymerase chain reaction (PCR) for the amplification of the 132 bp repeat region provided evidence for the presence of MDV-1 DNA in tumor tissue from several diseased turkeys. No evidence was found for the presence of REV.

Key words: MDV; PCR; turkeys

Introduction

MD is a lymphoproliferative disease of chickens caused by an alphaherpesvirus. It is characterised by an initial lytic process, subsequent mononuclear cell infiltration and finally induction of lymphomas in various organs.

Although chickens are the natural host, there are reports on the susceptibility of other avian species e.g. Japanese quail, pheasant, Great horned owl and turkey to MDV-1 (Jungherr, 1939; Halliwell 1970; Dutton *et al.*, 1973; Mohanty *et al.*, 1973; Powell and Rennie, 1984b). Several investigators have shown that turkeys are susceptible to experimentally induced MD (Sevoian *et al.*, 1963; Witter *et al.*, 1970; Witter *et al.*, 1974; Paul *et al.*, 1977; Elmubarak *et al.*, 1981; Powell *et al.*, 1984a). The gross lesions induced by MDV-1 in turkeys are most prevalent in liver and spleen. Other visceral organs and the peripheral nerves are infrequently involved. The pathogenesis of MD in turkeys has been shown to be similar to that in chickens (Elmubarak *et al.*, 1981; Nazerian and Sharma, 1984). However, transformed cells in turkeys were of B- and T-lymphocyte type (Elmubarak *et al.*, 1981; Nazerian *et al.*, 1982; Powell *et al.*, 1984a).

The first description of a nonexperimental MD-like condition in turkeys was provided by Andrewes and Glover (1939) in the UK. Other natural cases of lymphoma in turkeys were reported in Florida (Simpson *et al.*, 1957; Busch, 1970) and the Netherlands (Voüte and Wagenaar-Schaafsma, 1974).

Retrospectively, it is not possible to ascertain whether the causative agent of the described cases was MDV-1, the lymphoproliferative disease virus of turkeys (LPDV) or REV. The discussion on the natural occurence of MD in turkeys has become increasingly interesting following reports on

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MDV-1 field cases in France, Israel and Poland in recent years (Coudert *et al.*, 1995; Davidson *et al.*, 1996; Kozdrun personal communication, 1998).

Our objective was to diagnose the aetiology of a tumorous disease in turkeys on three farms in Germany. Attempts to isolate pathogenic virus in different cell culture systems failed, serological tests were inconclusive. To determine the causative agent of the disease, we decided to use PCR as described by Silva (1992).

Materials and Methods

Preparation of DNA samples. DNA was extracted from buffy coat cells, tumor and normal tissues by phenol-chloroform extraction described by Jux (1994). As positive controls for the PCR we used DNA from three strains of MDV-1. Turkey-MDV (T-MDV) DNA was received from Dr. I. Davidson, Israel and GA DNA (*Eco*RI-F and -X fragments) was received from Dr. R.L. Witter, USA. The spleen of a chicken experimentally infected with oncogenic MDV strain Italy (LAH, Cuxhaven) was received from Dr. C. Grund, Munich. As positive specificity controls we used DNAs from a vaccinal strain of HVT (LAH, Cuxhaven) in the 3rd passage on CEFs and MDV-2 SB1 strain (Intervet, Boxmeer) in the 4th passage on CEFs.

Oligonucleotid primers used in our study have been described previously (Silva, 1992) and detected MDV DNA by amplification of the 132 bp repeat region. The direct primer was 5'TGCGATGAA AGTGCTATGGAGG (22-mer) and the reverse primer was 5'GAGAATCCCTATGAGAAAGCGC (22-mer).

PCR. The reaction mixture (50 µl) contained 10 mmol/l Tris-HCl (pH 8.5), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 µmol/l dNTPs, 60 pmoles of each primer, 0.5 U of Tfl polymerase and 200 ng of template DNA. Following an initial melting step at 95°C for 1 min, 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 3 mins were carried out, followed by a final elongation step at 72°C for 7 mins. Mixtures lacking template DNA were routinely included as negative controls.

Electrophoresis of PCR products. Twelve µl of each reaction mixture was mixed with 3 µl of gel loading buffer. The samples were separated on a 2% agarose gel containing 0.5 µg/ml ethidium bromide in Tris-acetate-EDTA-buffer for 1 hr at 80 V. The sizes of the amplified DNA fragments were determined by comparison with a 100 bp DNA ladder.

Results and Discussion

Turkeys from the following farms were examined in our study:

Farm A: At this farm turkeys were kept from one-day-old, reared up to an age of 6 weeks and then sold to private persons. They were living near young hens, broilers, ducks, geese and guinea fowl. Three to four crops per year were produced. First cases of tumorous lesions were seen in 1994, the first poults died at an age of 7 weeks and the mortality rate reached 60%. The consulted veterinarian diagnosed "leukosis". In 1995 2/4 flocks were affected. In July 1996, the mortality rate reached 30% and the farmer consulted the Poultry Health Service of the Justus Liebig University. Sick birds were pale, unable to move and died 2 weeks after the first clinical signs appeared. Older, well developed birds often died with no clear signs of illness. Necropsied birds had solid tumors or diffuse infiltration in liver, spleen and kidney. The organs were often enlarged sometimes to several times of normal size. Sporadically the thyroid gland, intestine, thymus, adrenal gland and heart were affected. One bird had small tumors associated with subcutaneous nerves and skin lesions. Histological examinations showed a pleomorphic cell infiltration with mainly mononuclear cells in the affected organs. Neural lesions were rare and of the B- or C-type.

Farm B: 14 turkeys were purchased from a poultry merchant at an age of 4 weeks. It was the first time that turkeys were at this farm, no other poultry was kept there. The birds were maintained under free-range conditions. The first signs of disease were noticed at 8 weeks of age. The affected bird was apathic and prefered to sit, it died 4 weeks later. Two birds were necropsied at 18 weeks of age and blood samples were collected from the other turkeys. Liver and spleen of the necropsied turkeys were enlarged and had multiple nodular tumors. Lymphomas were also found in the kidney and one bird had a small solid tumor in the pancreas. Histological examination showed pleomorphic cell infiltration, mainly of blastoid type in the liver, spleen, kidney and thyreoid gland. The sciatic nerve of one bird showed lesions similar to type B.

Farm C: 10 turkeys were kept under free range conditions. Birds became sick at an age of 10 weeks, they were pale and reluctant to move. One bird was necropsied at the Veterinary Investigation Centre in Kassel and liver, kidney, spleen and EDTA-blood were sent to our institute. Histological findings consisted of pleomorphic cells in the liver, kidney and spleen.

To determine the causative agent of this tumorous disease, we inoculated CEFs and CEKs (Schat and Purchase, 1989) with tissue samples and buffy coat cells. Only HVT

Table 1. Serological results

Farm	No. of samples	MDV* AGP	HVT VNT	LLV* ELISA	REV* ELISA
A	12	negative	nd	negative	negative
	10	negative	positive	negative	negative
B	9	negative	nd	negative	negative
C	1	negative	nd	negative	negative

*Tests performed by Veterinary Laboratory, Lohmann Tierzucht, Cuxhaven. nd = not done.

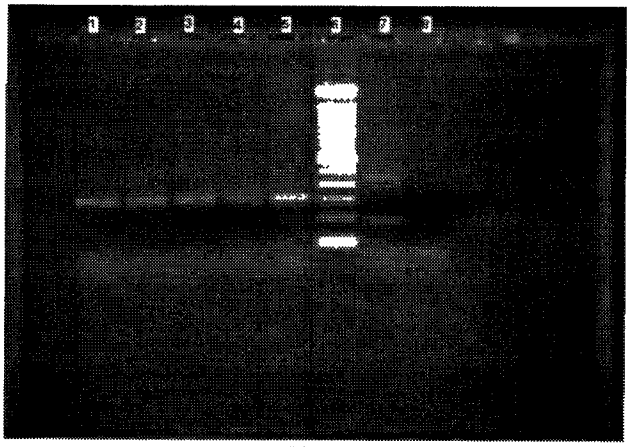

Fig. 1
PCR analysis of DNA extracted from blood and organ tissues of sick turkey from farm A
Liver (lane 1), blood (lane 2), heart (lane 3), spleen (lane 4), lung (lane 5), 100-bp ladder marker (lane 6), positive control, GA DNA, (lane 7), and negative control, no DNA, (lane 8).

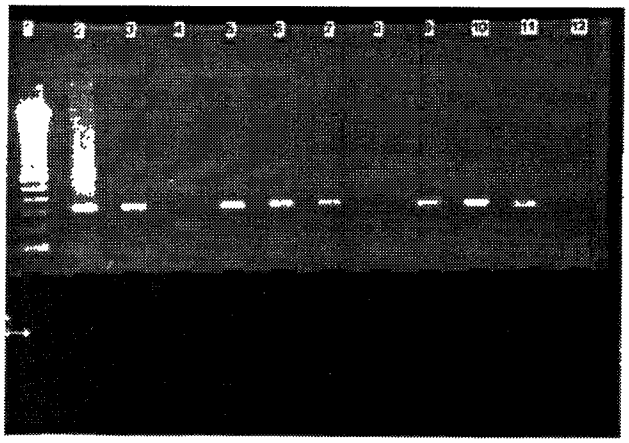

Fig. 2
PCR analysis of DNA extracted from organ tissues of three turkeys from farms A and C
Marker 100 bp ladder (lane 1), T-MDV (lane 2), Italy isolate (lane 3), GA MDV (lane 4), KK22/97, liver (lane 5), KK22/97, kidney (lane 6), KK22/97, thyroid gland (lane 7), KK1/97 spleen (lane 8), 2277/97, kidney (lane 9), 2277/97, spleen (lane 10), 2277/97, liver (lane 11), and negative control, no DNA (lane 12).

was isolated from the buffy coat cells in both cell culture systems. To keep HVT from overgrowing the cell culture vessel, we overlayed the cells with agar and punched out suspect small plaques. However, no virus other than HVT was detected. Virus neutralization test (VNT) showed the presence of antibodies against HVT. No antibodies were detectable against MDV-1, LLV or REV by agar gel precipitation test (AGP) or enzyme-linked immunosorbent assay (ELISA) (Table 1). Histological examination of the affected organs showed infiltration with pleomorphic cells, mainly lymphocytes and lymphoblasts.

No aetiological diagnosis was possible on the basis of these results. We therefore used PCR to search for the causative agent of the disease. Silva (1992) established a PCR procedure to differentiate pathogenic and non-pathogenic MDV-1. This method is based on the finding that pathogenic (MD+) and nonpathogenic (MD–) MDV-1 differs in the copy number of the 132 bp repeat region (Silva and Witter, 1985; Maotani *et al.*, 1986). The 132 bp repeat region is located in the terminal repeat (TR_L) and internal repeat (IR_L) flanking the unique long region (U_L). The MD+ genome contains one to three copies of these repeats, MD- three to a hundred. Choosing the oligonucleotide primers from sequences that flank the 132 bp repeat regions makes it possible to determine the number of copies present in the examinated DNA. Using the primers described above, the expected amplification products are 185, 317 or 449 bp in MD+. PCR products of MD– would be a series of bands with various sizes corresponding to the 132 bp repeat region (Silva, 1992). Another possibility is to choose primers mapping within the MDV-1 glycoprotein C gene (Zhu *et al.*, 1992). However, this method only differentiates between the serotypes 1, 2 and 3. It does not indicate the pathogenicity of the detected MDV-1. Amplification of the T-MDV DNA and the DNA extracted from the spleen of a chicken infected with MDV-1 strain Italy as described above gave a PCR product of 317 bp, representing two copies of the 132 bp repeat region. GA-DNA digested with *Eco*RI showed amplification products with one and three copies (185 bp and 449 bp) of the repeats. In order to test the specifity of the PCR, DNA of MDV-2 and HVT was used. No amplification was detectable.

DNAs of various tissues and buffy coat cells of the affected birds were used as template. We found amplification products of 317 bp (Figs. 1 and 2). Table 2 shows the organs which were positive in the PCR. Buffy coat cells from 9 birds from farm B were tested for MDV-1. Only one was positive. Tissue samples from two of these birds were positive, while the buffy coat cells were negative. This indicates low levels of viremia. Several authors have reported that turkeys inoculated with MDV-1 became persistently viremic and that levels of detectable circulating MDV-1 were generally lower than those in similarly inoculated chickens (Elmubarak *et al.*, 1981; Paul *et al.*, 1977; Powell *et al.*, 1984a; Powell and Rennie, 1984b). Witter *et al.* (1970) reported that their attemps to reisolate virus were unsuccessful.

The negative result of the MDV-1 AGP is not surprising, Witter *et al.* (1970) and Paul *et al.* (1977) recognized that experimentally inoculated turkeys developed no precipitating antibodies against MDV. Low levels of antibodies against

Table 2. MDV-1-positive organs by PCR

Farm	Animal No.	Organs
A	196/98	Liver, spleen, heart, lung, blood cells
	KK22/97	Liver, kidney, thyroid gland
B	2291/1/97	Liver, spleen, thymus
	2291/2/97	Liver, spleen
	2291/3/97	Buffy coat cells
C	2277/97	Liver, spleen, kidney, buffy coat cells

MDV-1 were detectable by direct immunofluorescence (Witter *et al.*, 1974).

In order to test whether vaccination against MDV would protect turkeys, Elmubarak *et al.* (1982) vaccinated turkey poults with HVT or attenuated JM. Later the poults were exposed to the pathogenic GA strain. The results demonstrated that this method of vaccination failed to protect turkeys against MD. They reported an age-related resistance to MD in turkeys at four weeks of age. Nazerian and Sharma (1984) demonstrated a negligible protection with HVT-vaccination against MDV-1 challenge. In 1997, turkeys from farm A were reared for the first 5–6 weeks of age in another (chicken-free) environment and were vaccinated twice with CVI 988 and HVT. Only one bird of 4 crops developed disease and showed tumorous lesions as described. The question, why turkeys have apparently become more susceptible to natural infection with MDV-1 and developed clinical disease is difficult to answer. The detection of pathogenic MDV-1-specific DNA from the tissue and buffy coat cells contradicts the hypothesis that a HVT mutation could be the aetiological cause. This rise in the number of MD cases in turkeys parallels the increased detection of highly oncogenic MDV-1 strains in recent years (Witter 1997). These isolates have been designated as very virulent+ (vv+) MDV-1 pathotype. They produce significantly higher levels of MD in HVT- and SB1-vaccinated chickens than the known vv MDV-1 strains. Another possible explanation for the rise in MD cases in turkeys could be a genetic change in the turkeys. Nazerian and Sharma (1984) compared the susceptibility of two different types of turkeys to MD and found the small white Beltsville turkeys to be resistant, while industrial type Nicholas turkeys were susceptible. The turkeys at farm A were Big 6 type, nothing is known about the genetic backround of the birds of farm B and C.

It appears so far that only small populations of turkeys under non-industrial farming conditions are affected in Germany. The infection probably spreads horizontally from MDV-1-infected chickens.

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