Evaluation of liver and heart lesions induced by experimental fowl adenovirus-4 infection in broilers and virus detection by immunohistochemistry, immunofluorescence and \textit{in situ} PCR.

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SUMMARY

The aim of this study was to compare the efficiency of immunohistochemistry, immunofluorescence and \textit{in situ} PCR to detect viral particles in liver and heart lesions experimentally induced by FadV-4 (Fowl Adenovirus 4) inoculation in broilers. For that, 4 week old healthy broilers were inoculated by FadV-4 (0.5 mL, EID$\log_{10}$ 7.3 (Chicken Embryo Infective Dose)/mL) orally (group I, n = 18) or intramuscularly (group II, n = 18) and 12 birds were not inoculated and served as negative controls. Birds were sacrificed 3, 6, 9, 12, 14 and 17 days after; necropsies were systematically performed and liver and heart samples were analysed for histopathology, and for detection of viral antigens using anti FadV-4 polyclonal chicken antibodies and revelation of the formed immune complexes by rabbit anti chicken IgG and biotinylated goat anti rabbit IgG and horse radish peroxidase (immunohistochemistry) or fluorosothiocyanate conjugated IgG (immunofluorescence) and using \textit{in situ} PCR throughout specific amplification of the hexon. Although no mortality was noted, oral and intramuscular inoculations have induced degenerative and inflammatory lesions in heart and liver and the intensity and duration of the organ damage were greater in intramuscularly infected birds. Immunohistochemistry evidenced viral particles in hepatocytes and in some Kupffer cells in liver and in cardiomyocytes in heart as well as in endothelial and mononuclear inflammatory (mainly lymphocytes) cells in all infected birds and the labelling intensity was well correlated to the lesion intensity whereas using immunofluorescence, the antigen distribution was revealed the virus presence in the same type cells and PCR positivity intensity whereas using immunofluorescence, the antigen distribution was well correlated to the disease extension and suggested that it may be useful to evidence latent infections.

**Keywords:** Fowl adenovirus 4, broiler, heart, liver, histopathology, immunohistochemistry, immunofluorescence, \textit{in situ} PCR, viral detection.

RÉSUMÉ

Evaluation des lésions hépatiques et cardiaques chez le poulet lors d’une infection expérimentale par l’adénovirus aviaire de type 4 et détection du virus par immunohistochimie, immunofluorescence et PCR \textit{in situ}.

Le but de cette étude a été de comparer l’efficacité des méthodes immunohistochimique et immunofluorescente et par PCR \textit{in situ} dans la détection des particules virales dans les lésions hépatiques et cardiaques induites expérimentalement par une infection par l’adénovirus aviaire de type 4 chez le poulet. Pour cela, des poulets sains âgés de 4 semaines ont été inoculés par le virus (0.5 mL, EID$\log_{10}$ 7.3 (Chicken Embryo Infective Dose)/mL) par voie soit orale (groupe I, n = 18) soit intramusculaire (groupe II, n = 18) et 12 oiseaux n’ont pas été inoculés et ont servi de témoins négatifs. Les oiseaux ont été sacrifiés 3, 6, 9, 12, 14 et 17 jours après inoculation et ont été systématiquement autopsiés ; les échantillons de foie et de cœur ont été recueillis pour une analyse histopathologique et pour détecter la présence des antigènes viraux capables de réagir avec des anticorps polyclonaux de poulets dirigés contre l’adénovirus aviaire de type 4 et révélation des particules virales dans les hépatocytes et dans quelques cellules de Kupffer dans le foie, dans les cardiomyocytes dans le cœur ainsi que dans les cellules endothéliales et dans les cellules inflammatoryes mononucléées (essentiellement des lymphocytes) chez tous les animaux infectés ; l’intensité du marquage a, de plus, été bien corrélé à l’intensité des lésions alors que par immunofluorescence, la distribution des antigènes viraux a été plus tardivement révélée et est apparue plus discrète dans les 2 organes. La PCR \textit{in situ} a aussi révélé la présence du virus dans les mêmes types cellulaires et l’intensité du marquage a été fortement associée à l’intensité des lésions, corroborant fortement les données immunohistochimiques. Ces résultats montrent que la PCR \textit{in situ} permet de détecter le virus en fonction de l’extension des lésions induites et suggèrent qu’elle peut être très utile dans le dépistage d’infections latentes.

**Mots-clés :** Adénovirus aviaire de type 4, poulet, cœur, foie, histopathologie, immunohistochimie, immunofluorescence, PCR \textit{in situ}, détection virale.
Introduction

It is known that some of fowl adenovirus (FadV) (serotypes 2, 4, 6, 8 and 10) of group-I avian adenovirus may induce severe lesions in liver and in heart, especially in chicken. In this regard, the disease is so called as Inclusion Body Hepatitis / Hydropericardium Syndrome (IBH/HPS) [2, 3, 10, 21, 24]. The infection commonly appears on 3-7 weeks old broiler during a period of self immune system activation and intense weight growth [13, 18, 23-25]. The infection can come out either solely or with other diseases such as Gumboro disease, Chicken Infectious Anaemia etc. [17, 24] which may depreciate the immune system and/ or when stress factors are exacerbated. Mortalities initiate suddenly and rapidly peak and afterward decrease in one or two weeks [1, 17, 24].

About experimental infections, it is generally preferred oral and parenteral (intramuscular, subcutaneous, intraperitoneal or ocular) routes. Incubation period can vary meanly from 2 to 5 days [7, 16, 32]. Macroscopically, it is noticed tan/dark yellow coloured transudate in pericardial sac and epicardial petechial haemorrhages. Moreover, apex of heart is sometimes turned likely to conic in shape due to hypertrophy [9, 19]. Liver is generally congested and swollen. In some severe cases, petechial or ecchymotic haemorrhagic areas are developed in subcapsular region of liver and a mottled general appearance is observed [9, 11]. Histopathologically, an important and characteristic sign of the infection is basophilic inclusion bodies found especially in nuclei of hepatocytes which appear large and round in shape or amorphous and which are surrounded by a noticeable clear, not stained, halo [8, 9, 11]. Besides, hydropic or vacuolar degeneration and necrosis and focal to diffuse mononuclear cell infiltration in portal regions are often noticed in parallel [8, 16, 19] and sometimes cholecytitis-cholangitis may be associated [29]. Heart lesions consisting in parenchyma or hyaline degeneration to necrotic changes in myocytes and focal haemorrhagic area particularly in severe cases are mostly developed in myocardium [16, 19, 22]. In addition, mononuclear cell infiltrations and degenerative changes which is localized in intimae and adventia layers of arterioles and veins can be seen [9].

On the other hand, many immunohistochemical studies (both immunoperoxidase and immunofluorescence methods) have been conducted for detecting different avian adenovirus serotypes in SPF (specific pathogen free) or healthy broilers of various ages, infected via different infection routes [26, 28]. In these studies, positive immunolabelling is generally found in hepatocytes. Additionally, molecular diagnosis methods are also employed which can detect in tissues adenoviral DNAs from different types and groups by in situ hybridization [14, 34]. However, in situ PCR methods are not up to now validated.

In the study, the development of heart and liver lesions coupled to direct viral antigen detection using immunohistochemical methods and in situ PCR method were purposed in not immuno-deficient healthy 4 week old broiler chickens infected by FadV-4 via oral and intramuscular routes. It is considered by immunoperoxidase method conspicuous reaction than by the immunofluorescence method. However, it is obtained by in situ PCR method more reliable results than by immunohistochemistry.

Material and Methods

ANIMALS AND PROTOCOL DESIGN

A total of 48 healthy Ross 308 broiler chickens, 4 week old, provided by the department of Zootechny, Faculty of Agriculture, Ankara University, were used: among them, 18 were infected via oral route (group I) and 18 were intramuscularly injected (group II) by 0.5 mL of FadV-4 viral suspension (EID\textsubscript{0} = \text{10}\textsuperscript{5.5-11} (Chicken Embryo Infective Dose)/mL, provided from Bornova Veterinary Control Institute of Ministry of Food, Agriculture and Livestock, İzmir, Turkey). The other 12 birds served as negative controls (group III) and received only PBS (Phosphate Buffer Saline pH 7.4, orally (n = 6) and intramuscularly (n = 6).

This study was approved by the Local Committee on the Ethics of Animal Experiments of the Ankara University.

GROSS EXAMINATION AND HISTOPATHOLOGICAL ANALYSIS

Necropsies were performed 3, 6, 9, 12, 14 and 17 days after treatments on 3 birds per time point in the infected groups and in one control bird at each date. Broilers were euthanized after suitable process anaesthesia (Pentothal (Thiopental) 5.5-11 mg/kg via intravenous route), cervical dislocation and decapitation. Livers and hearts were carefully observed.

Tissue (liver and heart) samples were fixed in 10% neutral formalin (pH 7.4) and were then routinely processed. After embedding in paraffin, tissue sections were cut at 5 µm from paraffin blocks and stained with haematoxylin-eosin then with Feulgen stain for visualising inclusion bodies.

VIRAL ANTIGEN DETECTION

Immunoperoxidase method

Tissue sections, which were taken on adhesive slides, were digested with 0.1% trypsin at 37°C for 10 minutes after deparaffinization and dehydration. Probable endogenous peroxidase activity in tissue was blocked by 3% hydroperoxide-methanol mixture at room temperature for 20 minutes. Protein blocking was performed using protein blocking sera (Dako) at 37°C for 30 minutes. The sections were incubated with anti FadV-4 primary chicken immune serum diluted at 1:40 at 37°C for 1 hour. After that, sections
were incubated at 37°C for 1 hour with rabbit anti chicken IgG (Sigma) diluted at 1:50, biotinylated goat anti rabbit IgG and horse radish peroxidase (HRP) marked antibody (Dako, ABC kit), consecutively (Avidin Biotin Complex Peroxidase (ABC-P) method). Tissue sections were then holding in 3-amino-9-ethylcarbazole (AEC) (Dako). For counterstaining, sections were kept in Mayer haematoxylin for 1 minute and mounted with glycerol and cover slip.

**Immunofluorescence method**

After deparaffinization-dehydration, trypsin action, protein blocking and primary serum incubation performed as described before, tissue sections were incubated with Fluoroisothiocyanate (FITC) conjugated IgG (Sigma) at 37°C for 1 hour and were mounted with glycerol and cover slip.

**In situ PCR**

Tissue sections taken onto adhesive slides were deparaffinized and rehydrated in deionized water. The sections were digested with proteinase K (6 μg/mL, Sigma) and ventilated at room temperature for 1 hour after acetylation with 0.3% acetic acid. After washing 2 times with Tris-HCl for 5 minutes, frames were placed onto glass slides and 50 µL PCR mixture (5 µL 10X PCR Buffer, 3 µL 50 mM MgCl₂, 4 µL 10 mM dNTP, 5 µL 0.4 mM dig-dUTP, 5 µL 10 mM forward primer H3 (5’ AACGTCAACCCCTTCAACCA CC3’), 5 µL 10 mM reverse primer H4 (5’ TTGCCTGTGGCGAAAGGCG 3’) [31], 2.3 µL of 2.5 U/µL i-Taq DNA Polymerase (Intron Biotechnology) and Jump Start Taq DNA Polymerase (Sigma), 20.7 µL DEPC (Diethyl Pyrocarbonate)) were added to each slides and then slides were cover tripped (Geneframe, Frames and coverslips, Abgene). The following cycle profile was applied in in situ PCR Thermal Cycler (Omnislide, ThermoScientific): only once predenaturation at 95°C for 7 minutes, denaturation at 95°C for 5 minutes for the first cycle and for 1 minute for the 24 other cycles, hybridation and extension for each cycle at 57°C for 90 seconds and at 74°C for 90 seconds, respectively, a final extension at 74°C for 2 minutes and the reaction was stopped at 30°C for 10 seconds. Slides were holding at 20X SSC (Standard Saline Citrate) for 1 minute.

For postfixation stage, slides were rehydrated in absolute ethanol and subsequently in degraded alcohols and after incubation with anti-digoxigenin-AP (Alkaline Phosphatase) (Fab fragment, Roche, diluted to 1:200) at 37°C for 1 hour, endogenous phosphatase activity in tissues was removed by 0.2 N HCl. As chromogen, NBT/BCIP (Nitro Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate) was selected at room temperature for 45 minutes. For counterstaining, the slides were stained with 0.1% Nuclear Fast Red for 3 minutes. The sections were mounted by using glycerol and cover slip. Control sections of the in situ PCR consisted in omission of Taq polymerase or primers in the PCR mixture and in omission of AP or conjugated anti-digoxigenin antibody during the revelation step.

All slides were observed under light microscope (Leica, DM4000B) and imaged by camera attachment (DFC-20). For fluorescence observation, a fluorescent microscope coupled to a photo attachment (Department of Pathology, Ankara University Faculty of Veterinary Medicine) was used.

**Results**

In orally or intramuscular infected chickens (groups I and II), livers were congested and mottled in appearance (figure 1). In addition, endocardium in especially both ventricles was haemorrhagic and in some cases, pericardial fluid accumulation was clearly viewed (figure 2). Endocardial petechias were found since the 3rd day after viral inoculation when intramuscular route was used (figure 3) and exacerbation of heart and liver injury was evident on day 6 in the group II. Any pathological findings were observed in the controls (not infected birds) during the experiment.
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### Table I: Liver and heart histopathological scores in FAdV-4 infected broiler chickens via oral (n = 18, group I) or intramuscular (n = 18, group II) routes and in the not inoculated controls (n = 12, group III) according to time after treatments (3 birds in each infected group and 2 one control birds were sacrificed per date).

<table>
<thead>
<tr>
<th>Control (group III)</th>
<th>Orally FadV-4 infected birds (group I)</th>
<th>Intramuscularly FadV-4 infected birds (group II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>++ (3/3) / IIB (2/3)</td>
<td>+ (2/3), ++ (2/3), +++ (3/3)</td>
</tr>
<tr>
<td>Day 6</td>
<td>++ (3/3) / IIB (2/3)</td>
<td>+ (2/3), ++ (1/3) / IIB (1/3)</td>
</tr>
<tr>
<td>Day 9</td>
<td>++ (3/3) / IIB (3/3)</td>
<td>++ (2/3), +++ (1/3) / IIB (1/3)</td>
</tr>
<tr>
<td>Day 12</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), +++ (2/3)</td>
</tr>
<tr>
<td>Day 14</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), ++ (2/3) / IIB (3/3)</td>
</tr>
<tr>
<td>Day 17</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), +++ (2/3)</td>
</tr>
</tbody>
</table>

- **no lesion:** no viral antigen in any cell; **+: weak positivity:** focal cell positivity; **++:** Mild positivity: positivity in different kind cells and in several foci; **+++:** moderate positivity: diffuse positivity in different kind cells and in nearly all foci.

### Table II: Liver and heart histochemical scores in FAdV-4 infected broiler chickens via oral (n = 18, group I) or intramuscular (n = 18, group II) routes and in the not inoculated controls (n = 12, group III) according to time after treatments (3 birds in each infected group and 2 one control birds were sacrificed per date).

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<td>+ (2/3), ++ (1/3) / IIB (1/3)</td>
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<tr>
<td>Day 9</td>
<td>++ (3/3) / IIB (3/3)</td>
<td>++ (2/3), +++ (1/3) / IIB (1/3)</td>
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<td>Day 12</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), +++ (2/3)</td>
</tr>
<tr>
<td>Day 14</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), ++ (2/3) / IIB (3/3)</td>
</tr>
<tr>
<td>Day 17</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), +++ (2/3)</td>
</tr>
</tbody>
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- **no lesion:** no viral antigen in any cell; **+: weak positivity:** focal cell positivity; **++:** Mild positivity: positivity in different kind cells and in several foci; **+++:** moderate positivity: diffuse positivity in different kind cells and in nearly all foci.

### Table III: Liver and heart in situ PCR results in FAdV-4 infected broiler chickens via oral (n = 18, group I) or intramuscular (n = 18, group II) routes and in the not inoculated controls (n = 12, group III) according to time after treatments (3 birds in each infected group and 2 one control birds were sacrificed per date).

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<td>+ (2/3), ++ (1/3) / IIB (1/3)</td>
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<td>++ (1/3), +++ (2/3)</td>
</tr>
<tr>
<td>Day 14</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), ++ (2/3) / IIB (3/3)</td>
</tr>
<tr>
<td>Day 17</td>
<td>++ (3/3) / IIB (1/3)</td>
<td>++ (1/3), +++ (2/3)</td>
</tr>
</tbody>
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- **no lesion:** no PCR reaction in any cell; **+: weak positivity:** focal cell positivity; **++:** Mild positivity: positivity in different kind cells and in several foci; **+++:** moderate positivity: diffuse positivity in different kind cells and in nearly all foci.
Histopathological scores according to time were summarized in Table I in orally and intramuscularly FadV-4 infected birds and in the not infected controls. Hyperaemia, oedema and hydropic degeneration and intranuclear inclusion bodies in hepatocytes were observed since the 3rd/6th days after orally viral inoculation (figure 4). Mononuclear cell infiltrations were noticed in portal region on day 9 and these lesions were encountered until the last day (day 17) of experiment. Hepatocyte inclusion bodies were obvious in broilers infected throughout the intramuscular route, particularly on days 3, 9 and 14. Furthermore, inflammatory changes, found especially at periphery of the v. centralis and in portal region, and hepatocyte degeneration were widespread and strongly evident throughout the whole experimental period. Hyaline degenerations in muscle bundles, lymphocyte infiltrates in interstitium and inclusion bodies in nuclei of myocardiocytes were evidenced since the 6th day in orally infected chickens. Necrosis and focal haemorrhagic areas developed on day 9 and all heart lesions persisted until the last day (day 17). When the virus was inoculated by the intramuscular route, degenerative and necrotic changes were mainly found on day 6 whereas inflammatory signs mononuclear cell (macrophages, lymphocytes and plasma cells) infiltrates and subendocardial haemorrhages and intima degeneration of some arterioles) were prominent since the 9th day (figure 5). Any histopathological findings were encountered in the negative controls.

Granular or diffuse immunostaining (immunoperoxidase method) in cytoplasm from mostly hepatocytes and from some Kupffer cells were evidenced since the 3rd day after oral or intramuscular viral inoculation (figure 6). Endothelial cells and mononuclear inflammatory cells also exhibited immunopositivity especially on day 9 in orally infected birds whereas in birds treated by the intramuscular route, endothelia in portal region were strongly immunolabelled from the 6th day to the 14th day (figure 6b) and positivity was also evidenced in biliary duct epithelium on day 17. In heart, viral labelling was found in cytoplasm and nucleus from endothelial cells lining the endocardium and diffusely in cytoplasm from some myocardiocytes and epicardial cells since the 3rd day in both infected groups. Thereafter, between days 6 and 9 (for group I) and 12 (for group II), the frequency of immunopositive cytoplasm and nucleus reactions in myocardiocytes (figure 7) as well as in epicardial and endothelial cells was increased. Additionally, immune reactions were noticed in endothelial cells lining arteriolar intimae in one orally infected broiler on day 14. The intensity of immunohistochemical scores according to time in the FAdV-4 infected and in the control birds is reported in Table II.

In liver, similar immunopositive results in infected birds were obtained with the immunofluorescence method but were slightly delayed (the immunolabelling was clearly evidenced on day 12 post infection in the group I and remained weak before) and slightly attenuated in group II.
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(figure 8). However in heart, immunofluorescence cytoplasm and nucleus positivity was restricted to myocardiocytes and was detected only since the 6th day (figure 9). No immune reaction with both methods was observed in the not infected control chickens.

In situ positive PCR was mostly localized in hepatocytes (cytoplasm and nucleus) around the v. centralis (figure 10) and in few Kupffer cells (cytoplasm) on day 3 after oral infection and thereafter (from the 6th day to the 17th day), positive reactions were also largely evidenced in hepatocytes in portal region (figure 11). Additionally, PCR products were also found in the cytoplasm from some endothelial cells of vessels since the 1st day post infection. In broilers inoculated by intramuscular route, the PCR labelling of hepatocytes and endothelial cells was similar but slightly attenuated on day 3 compared to birds inoculated orally. Furthermore, in this group, PCR products were also found in inflammatory cells infiltrating portal regions on day 12 (figure 12) and in some

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**Figure 6:** Immunopositivity in nuclei and cytoplasms of hepatocytes (black arrows), Kupffer’ cells (white arrows), endothelial cells from v. centralis and lining sinusoids (arrowheads) in broilers infected intramuscularly with FadV-4 (0.5 mL, EID50log2107.3 (Chicken Embryo Infective Dose)/mL) 3 days (a.) and 9 days (b.) after, X 400, ABC immunoperoxidase method.

**Figure 7:** Immunopositivity in cytoplasm of myocardiocytes (arrows), in a broiler infected intramuscularly with FadV-4 (0.5 mL, EID50log2107.3 (Chicken Embryo Infective Dose)/mL) 9 day after, X 400, ABC immunoperoxidase method.

**Figure 8:** Immunopositivity in nuclei and cytoplasms of hepatocytes (black arrows), Kupffer’ cells (white arrows), endothelial cells from v. centralis (arrowheads) in a broiler infected orally with FadV-4 (0.5 mL, EID50log2107.3 (Chicken Embryo Infective Dose)/mL) 12 days after, X 400, immunofluorescence method.

**Figure 9:** Immunopositivity in cytoplasm of myocardiocytes (arrows) in a broiler infected orally with FadV-4 (0.5 mL, EID50log2107.3 (Chicken Embryo Infective Dose)/mL) 6 days after, X 400, immunofluorescence method.
biliary duct epithelia on day 14. Diffuse PCR products were evidenced in cytoplasms and nuclei of myocardiocytes since the 3rd day in intramuscularly infected broilers and since the 6th day in orally infected ones (figure 13). Positive reactions were also observed in endothelial cells of intimae layer of arterioles since the 6th day in the group II (intramuscularly infected birds) and since the 14th day in the group I (orally infected birds) as well as in epicardial cells since the 12th day in the both infected groups. No positive PCR was seen in the control broilers. The PCR results were summarized in Table III.

Discussion

Up to date, it has been reported that serotypes 4, 5 and 8 of the group I avian adenoviruses (FAdV) caused IBH (Inclusion Body Hepatitis) in chickens and that alone the serotype 4 caused HPS (Hydropericardium Syndrome). In addition, several organs, especially liver and heart were affected [1, 10, 21, 24, 25]. It was stated that these syndromes occurred more commonly on 3 to 12 weeks old broilers in field studies and were carried out on embryos and on 1 to 10 days old chickens in experimental studies. The most possible route for developing the infection would be oral route as reported by SAIFUDDIN and WILKS [33]. In the present study, oral or intramuscular FadV-4 inoculation were efficient for developing the infection on 3 week old and healthy broiler chickens and have lead to similar signs to the natural infection. The incubation period varied from 6 to 15 days in naturally infections and from 48 to 72 hours, sometimes up to 7 days in experimental studies [5, 6, 32, 35] and the duration of the infection is generally reported as 2 or 3 weeks.
in 3–7 weeks old broiler chickens [15, 19, 24]. In this study, the incubation period was restricted to 3 days (72 hours) and the infection duration was at least 17 days. It is pointed out that many factors (virus serotype-strain, age and breeds of broilers or hens...) may influence the course (incubation and duration) of the disease and its severity (intensity of clinical signs and mortality rates) [12, 23]. Besides, several stress factors and the occurrence of immunosuppressive diseases such as IBD (Infectious Bursal Disease) and CIA (Chicken Infectious Anemia) were also involved [17, 24]. In this context, it would be a reason why 3–7 weeks old broilers may gain body weight and mortality rate may remain null or low despite intense lesions as it was observed in the present study when stress factors were limited and no immunosuppression was induced.

Macrosopically, livers are generally congested and mottled with discoloration and haemorrhages [10]. Prominent described findings in heart were the accumulation of transudate or exude in pericardial sac and a conic shape of apex [8, 9, 19]. In this study, these findings were clearly observed in both groups. However, the heart modifications tended to be alleviated as time passing suggesting that the infection slightly and gradually recovered.

Microscopically, intranuclear inclusion bodies, hydropic and vacuolar hepatocyte degeneration, mononuclear cell infiltration involving dominantly lymphocytes in portal regions and some hyperplasia of the Kupffer cells were generally evidenced in liver whereas necrotic areas were encountered in only a few cases [8, 11, 16, 19]. In heart, degeneration and necrosis [8, 16, 32] and hyalinisation [22] of myocardiocytes and oedema, haemorrhages and mononuclear cell infiltration in interstitium [9, 16] were described but were not systematically observed in infected birds. In the present study, liver and heart histopathological findings were present in orally and intramuscularly inoculated broilers and were in accordance with the literature [9, 22]. However, liver and heart injuries were more pronounced in intramuscularly inoculated birds since the 3rd day post inoculation while in orally infected birds, the lesions became intense around the 6th–9th days post inoculation.

Viral antigen distribution in several organs may be generally detected by immunoperoxidase method [3, 25-28, 33] but an immunofluorescence method is sometimes employed too [4, 30]. As reported, positive immunolabelling was mainly observed in hepatocytes and more rarely in Kupffer cells in the present study for the whole postinoculation period. In addition, positive reactions have also extended to the vascular endothelial cells and mononuclear cell infiltrations in portal regions since the 6th day after the viral inoculation. Positive immunolabelling in heart was generally localised to cytoplasms of myocardiocytes and remained moderate until the 9th day and the 6th day after inoculation by the oral and intramuscular routes respectively and increased after to the end of the postinoculation period. Using the immunofluorescence method, a diffusive green luminescence was observed in the cytoplasms of hepatocytes, Kupffer cells and myocardiocytes but the labelling intensity and the number of positive cells on the first days post inoculation appeared lower than with the immunoperoxidase method.

It was concluded that immunoperoxidase method gave more reliable results on antigenic distribution of virus than the immunofluorescence method.

Different loops of DNA of several avian adenoviruses may be detected by in situ hybridization method which resemble to in situ PCR method widely used in molecular pathology [16, 20]. However, hexon regions based on classification of avian adenovirus were merely studied on falcons and was encountered in mostly hepatocytes and biliary duct epitheliums [14, 34]. Taken into account these findings, this is the first study that reports the presence of FAdV-4 DNA fragments in hepatocytes (primary in the periphery of the v. centralis and secondary in periportal localisation) and in myocardiocytes. In addition, the presence of the virus gene was also detected in other cell types (Kupffer cells, endothelial cells and mononuclear inflammatory cells) in parallel to extension of histopathological lesions and to the viral antigen distribution (evidenced by the immunoperoxidase method). Consequently, the in situ PCR method may easily detect the presence of the virus by amplifying a specific DNA sequence in different cell types and tissues according to the disease extension. It is considered that the method may be useful to follow the infection progression or to evidence latent infections, especially in viral infections similar to avian adenovirus infections.

As a conclusion, the present results show that liver and heart lesions experimentally induced by FAdV-4 inoculation can develop in 4 weeks old chickens despite the activation of the immune system. Additionally, the use of the in situ PCR method in these kinds of inconspicuous or latent infections may give reliable results as immunohistochemistry by detecting a specific viral DNA sequence.

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