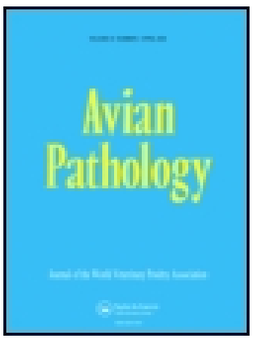

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Monitoring of pigeon paramyxovirus type-1 in organs of pigeons naturally infected with *Salmonella* Typhimurium

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An experimental pigeon paramyxovirus (pPMV-1) infection was followed by reverse transcription-nested polymerase chain reaction for 31 days after infection, in 16 organs of inoculated or contact pigeons naturally infected with *Salmonella* Typhimurium. With two exceptions, both groups presented similar results. Typical nervous signs and a green diarrhoea were observed. The spread of pPMV-1 was relatively quick, all organs being largely positive at 4 days after inoculation or contact. The lung, spleen, caecal tonsils, kidneys and brain, for which almost all tested samples remained positive during most of the experiment, seemed to be the principal targets for virus persistence. However, the virus was significantly recovered later in the brain parts and for longer in the trachea of the contact pigeons than of the inoculated ones.

Introduction

Avian paramyxovirus type-1 (aPMV-1), also known as Newcastle disease virus (NDV), is a member of the newly created genus *Avulavirus* within the viral family *Paramyxoviridae* (accepted proposal available on International Committee on Taxonomy for Viruses (ICTV) website: http://www.danforthcenter.org/iltab/ICTVnet). Virulent isolates cause Newcastle disease in poultry flocks, a highly contagious and economically important disease of poultry.

Pigeon paramyxovirus type-1 (pPMV-1) isolates are variants of aPMV-1 with few antigenic differences detected by monoclonal antibodies (Alexander *et al*., 1984a). pPMV-1 reached European racing and show pigeon lofts in 1981 (Biancifiori & Fioroni, 1983), was responsible for a true panzootic by 1985, and still remains enzootic for pigeons in most countries (Alexander, 2001). Chickens are susceptible to pPMV-1 experimental infections (Gelb *et al*., 1987; Parede & Young, 1990; King, 1996), but a natural Newcastle disease neurotropic form may also occur as demonstrated with pigeon variant outbreaks in commercial chickens seen in 1984 in Great Britain (Alexander *et al*., 1984b).

The persistence of the virus in targeted organs of pigeons has not been investigated, although such a phenomenon is documented for other paramyxoviridae (Wiman *et al*., 1998). In fact, previous published studies were restricted to following either virus cloacal shedding (Alexander & Parsons, 1984; Pearson *et al*., 1987) or virus detection in a few organs for a short time (Shirai *et al*., 1988) after pPMV-1 infection of pigeons, and investigators were hampered by the time/cost-consuming virus isolation method. In addition, in the field, pigeons can be infected with other pathogens such as *Salmonella*, circoviruses, herpesviruses, and the efficacy of vaccines has not been assessed under these conditions.

As an attempt to complete these data and to mimic a possible field situation, in the present work, the persistence of pPMV-1 was assessed for 5 weeks using a reverse transcription (RT)-nested polymerase chain reaction (PCR), in 16 organs of

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pigeons naturally infected by *Salmonella* Typhimurium and co-infected (either oculo-nasally or by contact) with a recent French pPMV-1 strain.

Materials and Methods

Virus

pPMV-1 strain 99299 was isolated according to reference methods (Anonymous, 1992; French COFRAC norms Pr112/00/VA10/00 and Pr112/00/VA30/00) in 1999 in France from an infected pigeon flock exhibiting green diarrhoea, torticolis and mortality. This strain displayed an Intra Cerebral Pathogenicity Index of 1.4. It was grouped in the P group of aPMV-1 as defined by Collins *et al*. (1989) using monoclonal antibodies. Its fusion cleavage motif was ^{112}RRQKR^{119}, and this strain was classified as a typical pPMV-1. So it was considered as representative of European recent isolates (Meulemans *et al*., 2002).

Animals

Weaned conventional hybrid 4-week-old meat pigeons were obtained from a commercial breeding in Brittany (France) with no recorded disease history. Parents were vaccinated against pigeon paramyxovirosis once a year with a commercial oil-adjuvated inactivated vaccine. During the 4-week quarantine of the young pigeons in our experimental biosafety BL3 containment facilities (inside, the animals were kept in 2 m^3 cages), *Salmonella* Typhimurium was isolated, using brilliant green Müller–Kauffmann tetrathionate broth for selective enrichment and XLT4 selective agar for isolation, from the trachea, lung and liver of three pigeons found dead, one of which having displayed respiratory problems (rales). In addition, 33% of the pigeons were seropositive for *Salmonella* Typhimurium using the enzyme-linked immunosorbent assay (ELISA) test mentioned later. Each pigeon was tested negative for Newcastle disease (aPMV-1) antibodies.

Experimental design

One hundred 8-week-old pigeons were used to form three groups in two separate biosafety BL3 containment facilities. The *Salmonella* Typhimurium-seropositive pigeons were randomly distributed between the three groups.

Forty-four pigeons were inoculated oculo-nasally with 10^6 median embryonated egg infectious doses (EID_{50}) in 0.1 ml per animal of 99299 isolate (inoculated group). Four days after inoculation (dpi), 38 uninoculated pigeons, referred to as the contact group, were mixed with the inoculated pigeons. The remaining 18 pigeons in the separate room remained uninoculated and formed the control group.

For 31 days, every 3 or 4 dpi (for the inoculated group) or days after contact (dpc) (for the contact group), three pigeons with signs of paramyxovirosis (when possible) were killed. Once a week, two pigeons were also killed in the control group. For each killed pigeon, blood and 16 organs (trachea, lung, duodenum, jejunum, ileum, cloaca, caecal tonsils, bursa of Fabricius, spleen, thymus, liver, pancreas, heart, kidney, cerebral hemisphere and cerebellum) were collected aseptically. A 9 mm^3 aliquot of each organ was stored at −70°C until analysis by RT-nested PCR. Blood samples were also taken from 10 inoculated and 10 contact pigeons at 10 and 21 dpi, and from control, inoculated and contact survivors at the end of the experiment.

Sera from blood samples were stored at −20°C for aPMV-1 and *Salmonella* Typhimurium antibody detection.

Virus genome detection

A RT-nested PCR (Barbezange & Jestin, 2002) was used to detect virus nucleic acids directly in organs. Briefly, RNA from a 9 mm^3 organ aliquot stored at −70°C in a 1.5 ml microtube (between 30 and 100 mg depending on the nature of the tissue) was extracted using 1 ml TRIzol® reagent (Gibco, Invitrogen) and Piston Pellet Bleu (Merck Eurolab) homogenizer. Reverse transcription was realized in a 20 µl reaction mix with hexamers and MMLV (Gibco, Invitrogen) according to the manufacturer's instructions. The first round and the second round of PCR were realized with AmpliTaqGold® DNA polymerase (Applied Biosystems) on a GeneAmp® 9600 thermocycler (Applied Biosystems), with Unpc-Lnph and Unpe-LnpK primer sets, respectively (1034 and 379 base pairs of nucleoprotein gene, respectively). Amplification products were detected by gel electrophoresis.

In a comparative study (Barbezange & Jestin, 2002) with the international reference virus isolation method using embryonated chicken eggs (Anonymous, 1992), the RT-nested PCR was shown to be as sensitive as virus isolation at the early stages of infection, and significantly more sensitive than virus isolation at the late stages of infection. At those late stages, the virus might not be fully infectious and it might be in a neutralized or inactivated form, which would, however, be detected by the RT-nested PCR.

aPMV-1 antibody detection

aPMV-1 antibodies were detected by a haemagglutination inhibition (HI) test according to French AFNOR norm NF U.47-011 (June 2000), using four haemagglutination units (HA) of the LaSota strain as antigen. The HI titre was the inverse of the highest dilution inhibiting the 4 HA and expressed as $\log_2$.

Typhimurium antibody detection

The ELISA technique used plates coated with *Salmonella* Typhimurium flagellin antigen and lipopolysaccharides (Proux *et al*., 1998).

Statistical analysis

Statistical significance was calculated at the 0.05 level of probability. Survival rates defined on intervals of 5 days were compared with the Actuarial method and the approached LogRank test. Analysis of variance was used to compare HI titres. Chi-square or Fisher exact tests according to the sampling size were used for *Salmonella* Typhimurium-positive percent comparison and for RT-nested PCR-positive number comparison. Analysis of variance, chi-square and Fisher exact tests were performed on SYSTAT® 7.0 software (SPSS Inc.)

Results

Signs

No pPMV-1 clinical signs were noticed in the control group pigeons. In the inoculated and contact groups, the observed clinical signs were consistent with those described in the literature for pigeons naturally or experimentally infected. For both groups, morbidity reached about 90%.

In the inoculated group, clinical signs were noticed as soon as 4 days after oculo-nasal inoculation and until the end of the experiment (31 dpi). The first sign was a decrease of activity resulting in apathy and depression. From 8 dpi, digestive and neurological signs appeared. The most noticeable sign was green diarrhoea, which became extremely watery about 15 dpi but almost disappeared after 24 dpi. Among neurological signs, drooping wings and head were first noticed, then lack of coordination and torticollis. The torticollis was still seen in the inoculated pigeons killed at 31 dpi. Moreover, other signs were observed in some pigeons, including ruffled feathers, tremors, oedematous eyelids, and loss of condition (amyotrophy and weight loss).

In the contact group, the same signs were observed from 4 dpc, but for a shorter period. Indeed, no marked sign was observed after 24 dpc.

No gross lesion was observed, irrespective of group.

Survival rates

Because pigeons were killed regularly throughout the trial, it was not possible to calculate mortality rates; however, the use of survival rate indices allowed us to take the killed pigeons into account. The contact group survival rate at the end of the experiment (60%) was lower than that in the inoculated group (76%). However, statistical comparison using the approached LogRank test (0.05 level of probability) showed no significant difference.

Serology

All pigeons tested during the quarantine were negative by HI test (titres < 1 $\log_2$) and were considered free of pPMV-1. After inoculation or contact, both groups had similar titres. Seroconversion was slow, and a mean titre above the positivity threshold of four was not seen until 3 weeks after infection (Figure 1). From the third week after inoculation or contact, the mean titres for both inoculated and contact groups ranged from 4.5 to 6.5 $\log_2$, whereas pigeons of the control group remained negative. The analysis of variance performed for each week showed no significant difference between inoculated and contact groups.

During the quarantine, 33% of the pigeons were found positive by the *Salmonella* Typhimurium ELISA. After inoculation and contact, 49 and 54% were positive in the control and exposed (inoculated plus contact) groups, respectively. Statistical comparison of these data by the chi-square test showed no significant difference between groups.

Virus genome distribution for the inoculated group

None of the Control pigeon organs was found positive for pPMV-1 genome by RT-nested PCR.

The RT-nested PCR allowed detection of the virus genome as soon as 2 dpi for trachea and lung samples (Figure 2). Nevertheless, while lung samples were found positive until the end of the experiment (31 dpi), the inoculated pPMV-1 was not detected in trachea samples after 14 dpi. The four digestive organs tested were found positive from 4 dpi, but cloacal samples were the only ones for which at least one sample out of the three was regularly positive after 17 dpi. Caecal tonsils and spleen were lymphoid organs positive from 2 to 31 dpi (Figure 2), while the bursa of Fabricius was positive until 28 dpi. In some cases the thymus had regressed and could not be examined. Among internal organs, the liver was positive from 4 to 14 dpi and the pancreas was generally positive from 4 to 10 dpi, whereas virus genome was detected in the heart, kidney, cerebral hemisphere and cerebellum until nearly the end of the experiment (Figure 2).

Thus, the lung, caecal tonsils, spleen and kidney were the organs that were consistently positive. On the contrary, the duodenum, jejunum, ileum, liver, pancreas and trachea were less frequently positive.

Virus genome distribution for the contact group

A similar distribution was obtained for most organs in the contact group, but significant differences were noticed for the trachea and central nervous system (Figure 2).

RT-nested PCR detected the virus genome in trachea samples collected until 28 dpc. In both parts of the central nervous system (cerebral hemi-

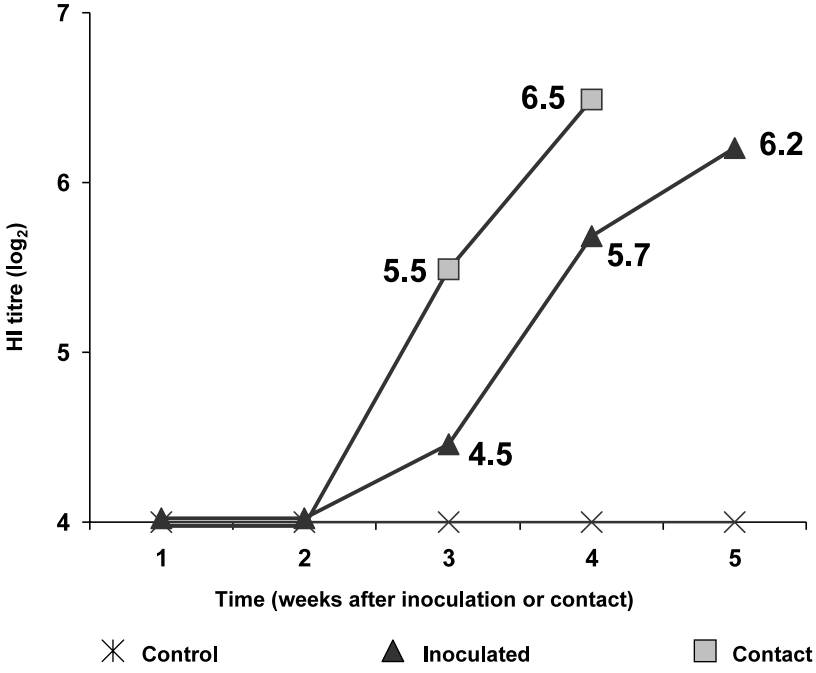

Figure 1. *Mean HI titres for the inoculated and contact groups. The positive threshold is 4 $\log_2$.*

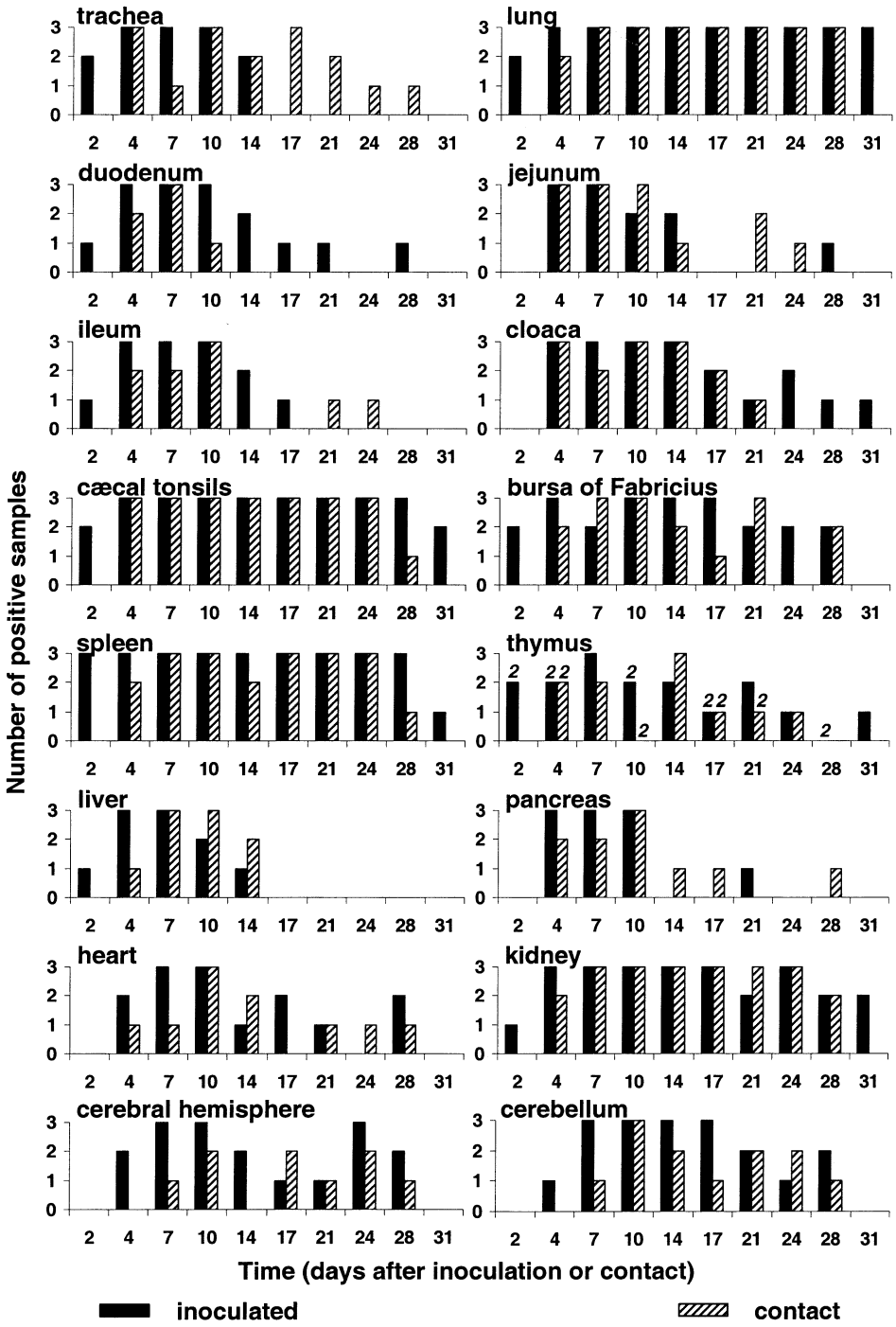

Figure 2. *Distribution of pPMV-1 by RT-nested PCR in the 16 collected organs for the inoculated and contact groups: number of positive samples out of three tested for each organ at each sampling time (no sampling for the contact group at 2 and 31 dpc). For the thymus, the number of samples tested (depending on whether or not the thymus was present) is indicated in italics above the histogram when differing from 3.*

sphere and cerebellum), pPMV-1 was not detected before 7 dpc. When combining data of the trachea and lung between 4 and 14 dpi/dpc, no significant difference between inoculated and contact groups was seen (Fisher exact test $P = 0.348$), whereas virus genome was significantly detected in more trachea samples for the contact group than for the inoculated group between 17 and 28 dpi/dpc (chi-square $P = 0.035$).

Similarly, for the combined data of both central nervous system parts between 4 and 7 dpi/dpc, virus genome detection was significantly lower in the contact group (Fisher exact test $P = 0.012$).

Discussion

The natural disease (torticollis and green diarrhoea) was successfully reproduced by the oculo-nasal inoculation of 8-week-old pigeons. Moreover, the virus was successfully transmitted to susceptible pigeons introduced in the same cage as the

inoculated ones, 4 days after inoculation, and the same signs were seen.

Alexander and Parsons (1984) did not observe clinical signs when they tried to reproduce pigeon paramyxovirus disease by intranasal inoculation or by contact. Nevertheless, they succeeded in transmitting the virus, as they isolated it from both intranasally inoculated and contact pigeons. Pearson *et al*. (1987) also failed to reproduce the disease by a natural route of inoculation, although El Mubarak *et al*. (1990) and Zanetti *et al*. (2001) observed signs when they inoculated conventional pigeons orally with pPMV-1, but no transmission by contact was attempted. However, none of these authors mentioned the sanitary status of their experimental pigeons. Alexander and Parsons (1984) hypothesized that co-infection with other infectious agents might be responsible for the development of the disease in the field, which could explain the failure to reproduce the clinical signs. At least some of the pigeons we used in this study could be considered as naturally infected with *Salmonella* Typhimurium, as confirmed by serological analysis for 54% of the animals and by bacterial analysis for three pigeons found dead during the quarantine. This might explain that we could observe signs of paramyxovirosis, although no particular relationship was noticed between the *Salmonella* Typhimurium-seropositive pigeons and those with paramyxovirosis signs. Some discrepancies exist between authors in the nature of signs of experimental pigeon salmonellosis reported. Vereecken *et al*. (2000) described mortality and diarrhoea, associated with large gross lesions in liver, intestine and kidneys, but others mainly described articulation problems (Proux *et al*., 1998). It should also be mentioned that the large greenish watery diarrhoea might be related to the presence of pPMV-1 in the digestive organs of most infected pigeons, since the diarrhoea was not detectable beyond 24 dpi, when the digestive organs were regularly negative using our RT-nested PCR. In addition, morbidity and mortality that were observed here agreed with data from studies of natural pPMV-1 infection (Alexander *et al*., 1984b; Tangredi, 1985), and all the signs we noticed have been described previously for other pPMV-1 strains (Alexander *et al*., 1984b; Tangredi, 1985, 1988).

Like most authors reporting experimental pPMV-1 infections (Barton *et al*., 1992; Johnston & Key, 1992; Lumeij & Stam, 1985; Maeda *et al*., 1987), we did not observe gross pathological changes, although Pearson *et al*. (1987) noticed haemorrhages on digestive organs and on pancreas, and El Mubarak *et al*. (1990) observed haemorrhages also on the brain.

The serological response to pPMV-1 reported here was low (maximum mean titre about 6.5 $\log_2$) and late (3 weeks after inoculation or contact), although at 2 weeks after inoculation we had already detected a positive antibody titre for some pigeons (six out of 13). This was in agreement with the results of Alexander and Parsons (1984), although at 2 weeks after inoculation they found four pigeons out of four with a positive HI titre (mean 5.3 $\log_2$) with an inoculated virus dose 100-fold higher than we used. We used the LaSota aPMV-1 strain as the antigen for the HI test, and higher titres might be detected with the homologous strain (Stone, 1989). However, Duchatel *et al*. (1985) obtained lower titres when they tested pPMV-1-infected pigeon sera against homologous antigen than against a classical aPMV-1 antigen. The increase of *Salmonella* Typhimurium-seropositive pigeons after infection (inoculation or contact) was not significant and might be explained by the stress linked to their new and confined environment.

To our knowledge, this is the first report monitoring, for as long as 31 days, the distribution of a pPMV-1 strain genome in as many as 16 organs of inoculated and contact pigeons demonstrated as naturally infected with *Salmonella* Typhimurium. In fact, several authors have followed pPMV-1 cloacal shedding only (using cloacal swabs and embryonated chicken eggs for virus isolation) after having inoculated, by different routes, pigeons of unknown sanitary status (Alexander & Parsons, 1984; Duchatel *et al*., 1985; Pearson *et al*., 1987). Alexander and Parsons (1984) and Pearson *et al*. (1987) were able to detect the inoculated pPMV-1 in cloacal swabs more than 3 weeks after inoculation or contact, whereas Duchatel *et al*. (1985) found only 10% of pigeons positive at 14 days after intramuscular and oculo-nasal inoculation. As we had agreement between cloaca tissue samples and cloacal swabs (data not shown), our results are more in agreement with the former studies, although virus strain, dose and route of inoculation were different.

We used a RT-nested PCR to detect virus genome, which had the advantage of being less consuming of bio-materials such as embryonated chicken eggs than the classical virus isolation technique. There is no specific requirement for weight of the organ sample to be processed by the reference virus isolation method. Consequently, the influence of the weight of organ samples processed by our RT-nested PCR was not investigated. Moreover, there is, at the moment, no RT-PCR officially recommended for diagnosis of aPMV-1 or pPMV-1 from clinical samples. The limits of our RT-nested PCR regarding the possible detection of inactivated viruses were previously highlighted, as was its possible improvement by targeting mRNA to demonstrate viral replication (Barbezange & Jestin, 2002).

The present virus distribution analysis, obtained by RT-nested PCR detection, allowed one to select organs of particular interest for monitoring pPMV-

1 infection after killing and bleeding, as it is routinely practised. For that purpose, the lungs, kidneys, spleen, caecal tonsils and central nervous system were selected. However, the natural infection of our pigeons with *Salmonella* Typhimurium on the one hand, and viraemia on the other hand, although transient according to studies on chicken infections with aPMV-1 (Shirai *et al*., 1988; Parede & Young, 1990), might have interacted with the observed pPMV-1 distribution. Thus, one could criticize our approach for pathogenesis purposes. However, the comparison with the contact group showed that the oculo-nasal route of inoculation was valid to reproduce the natural infection, as we obtained for this group almost the same distribution as for the inoculated group. Then, the long persistence of pPMV-1 genome in cerebral hemispheres was confirmed by virus isolation using the reference method with embryonated eggs (Barbezange & Jestin, 2002), and it might explain the predominance of neurological signs during pigeon paramyxovirosis.

Our experimental model that mimics natural conditions might help to improve experimental assessment of vaccine efficacy in order to better control paramyxovirosis. In addition, our model could also be a tool to evaluate dual infections, particularly with immunosuppressive viruses like pigeon circovirus (Todd, 2000).

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RÉSUMÉ

Monitoring du paramyxovirus du pigeon de type 1 dans les organes de pigeons infectés naturellement par *Salmonella Typhimurium*

Des pigeons naturellement infectés par *Salmonella Typhimurium* ont été inoculés avec un paramyxovirus du pigeon de type 1 (p PMV-1), un autre groupe de pigeons non inoculés a été maintenu à leur contact. Pendant 31 jours après inoculation ou contact des prélèvements de 16 organes ont été réalisés et analysés par RT-PCR nichée. Les résultats ont été similaires pour les groupes inoculés et contacts à l'exception de deux d'entre eux. Des symptômes nerveux et une diarrhée verte ont été observés. La dissémination du pPMV-1 a été relativement rapide, tous les organes ont été très positifs 4 jours après l'inoculation ou le contact. Les échantillons de poumons, rate, amygdales caecales, reins et cerveau ont été presque tous positifs durant l'expérimentation, et semblent être les principales cibles de la persistance du virus. Toutefois, le virus a été réisolé significativement plus tardivement après le contact dans certaines parties du cerveau et plus longtemps dans la trachée des pigeons contacts en comparaison de ceux inoculés.

ZUSAMMENFASSUNG

Überwachung einer Tauben-Paramyxovirus-Typ 1-Infektion in Organen von natürlich mit *Salmonella typhimurium* infizierten Tauben

Der Verlauf einer experimentellen Tauben-Paramyxovirus(pPMV-1)-Infektion wurde mittels der RT-nested PCR 31 Tage lang nach der Infektion in 16 verschiedenen Organen von mit dem Virus inokulierten Tauben oder Kontakttauben, die gleichzeitig auf natürliche Weise mit *Salmonella typhimurium* infiziert waren, verfolgt. Mit zwei Ausnahmen zeigten beide Gruppen ähnliche Ergebnisse. Typische nervale Symptome und grünlicher Durchfall wurden beobachtet. Die Ausbreitung des pPMV-1 im Körper ging relativ schnell, da alle Organe 4 Tage nach Inokulation oder Kontakt hochgradig positiv waren. Lunge, Milz, Zäkaltonsillen, Niere und Gehirn, das in fast allen getesteten Proben über den größten Teil des Experiments positiv blieb, schienen die Hauptzielorgane für die Viruspersistenz zu sein. Das Virus wurde jedoch in den Kontakttauben in Teilen des Gehirns später und in der Trachea länger entdeckt als bei den inokulierten Tauben.

RESUMEN

Monitorización del paramixovirus de la paloma tipo-1 en órganos de palomas infectadas con *Salmonella* Typhimurium

Se realizó una infección experimental con paramixovirus de la paloma (pPMV-1) seguido de una RT-PCR anidada durante 31 días tras la infección, en 16 órganos de palomas inoculadas o en contacto con palomas infectadas de forma natural con *Salmonella* Typhimurium. Excepto en dos casos, ambos grupos presentaron resultados similares. Se observaron los síntomas nerviosos típicos y diarrea verdosa. La diseminación de pPMV-1 fue relativamente rápida y todos los órganos resultaron positivos a los 4 días de la inoculación o contacto. Los principales órganos diana del virus que resultaron positivos durante prácticamente todo el experimento, fueron el pulmón, el bazo, las tonsilas cecales, los riñones y el encéfalo. No obstante, el virus fue recuperado significativamente más tarde del encéfalo y durante más tiempo de la tráquea en las palomas en contacto que en las que fueron inoculadas.