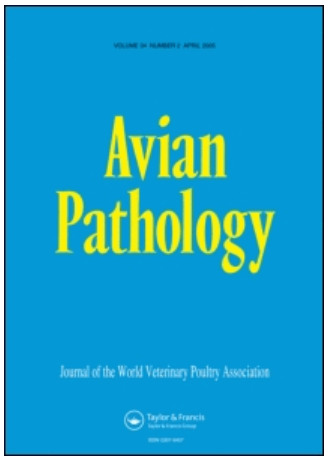

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Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens

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Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens

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Vaccinated and non-vaccinated specified pathogen-free White Leghorn laying chickens were challenged at peak of lay by the intravenous or oculonasal route with a virulent avian metapneumovirus (aMPV) subtype B chicken strain. Severe clinical signs and a drop in egg production were induced in the non-vaccinated intravenously challenged birds whereas the vaccinates were not affected. Live virus excretion was demonstrated in the faeces and respiratory tract of non-vaccinated hens for up to 7 days post intravenous challenge. After oculonasal challenge, virus excretion could only be demonstrated in the respiratory tract for up to 5 days. No live virus excretion was found in either the faeces or the respiratory tract of vaccinated birds. Concurrent with live virus isolation, the presence of viral RNA was demonstrated by single reverse transcription-polymerase chain reaction (RT-PCR). Nested RT-PCR was more sensitive and viral RNA could be detected in non-vaccinated birds up to 28 days post either intravenous or oculonasal challenge, at which time the experiment was terminated. Viral RNA was detected for up to 12 days in vaccinated birds. This is the first study investigating excretion of aMPV and viral RNA in vaccinated and non-vaccinated laying hens challenged under experimental conditions. The results are of importance with regard to the persistence of aMPV and the appropriate diagnostic detection method in laying birds.

Introduction

The genus avian *Metapneumovirus* (aMPV) includes different viruses isolated from various species, belonging to the subfamily *Pneumovirinae* within the *Paramaxoviridae* and so far known as avian pneumoviruses (Pringle, 1998). Based on neutralization patterns and sequence analysis, the *Metapneumoviruses* are currently separated into four different subgroups (A to D) (Bäyon-Auboyer *et al*., 2000).

First described in the late 1970s, for some time it was thought that *Metapneumoviruses* were solely a pathogen for turkeys (Buys & Du Preez, 1980). In this species the infection leads to a severe rhinotracheitis that can result in high production losses, especially if the initial viral infection is associated with *Mycoplasma gallisepticum*, *Bordetella avium* or pasteurella-like organisms (Cook *et al*., 1991; Naylor *et al*., 1992; Khehra *et al*., 1999). In laying turkeys the infection can result in a severe drop in egg production of up to 40% (Stuart, 1989).

In chickens, aMPV infection is often associated with the swollen head syndrome (O'Brien, 1985; Cook *et al*., 1988; Perelman *et al*., 1988; Jones *et al*., 1991). However, the disease could not be reproduced using aMPV alone and chicken flocks can be infected without showing clinical signs (Droual & Woolcock 1994; Al-Ankari *et al*., 2001). Even though a reduced laying performance is reported in broiler breeders in connection with swollen head syndrome (O'Brien, 1985; Perelman *et al*., 1988; Hafez & Löhren, 1990), the effect of aMPV on the reproductive tract is still not clear. Currently, in field investigations it is often assumed

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that a reduced egg production could be linked with an aMPV infection, without clear evidence based on relevant diagnostic methods. Experimental data in this area are limited to a few studies with different outcomes. Whereas Khehra & Jones (1999) and Majó *et al.* (1995) failed to demonstrate the replication of aMPV in the chicken oviduct, Cook *et al.* (2000) induced a severe drop in laying performance in specific pathogen-free (SPF) birds by intravenous application of a virulent aMPV isolate. Using this route of application it was possible to demonstrate the presence of aMPV in the oviduct by immunoperoxidase staining. In contrast, the oviduct of immature chickens was found unsusceptible after infecting young chicks (Catelli *et al*., 1998).

Using the same challenge model as described by Cook *et al.* (2000) the objective of this investigation is twofold. First, to investigate whether live virus or viral RNA is excreted via the oropharynx or cloaca following different challenge routes in fully susceptible laying chickens at peak of lay and, second, to investigate the influence of vaccination on virus excretion.

Materials and Methods

The experimental scheme reported by Cook *et al.* (2000) was used, except that an oculonasal challenge group was included at peak of lay. In addition, major attention was given to live virus or viral RNA excretion. Birds were swabbed from the pharynx and the cloaca in duplicate, and the two sets of swabs were processed for virus isolation and single or nested reverse transcription-polymerase chain reaction (RT-PCR).

Animal experiment

One-day-old White Leghorn SPF chicks were vaccinated against Marek's disease then divided into two groups designated A and B and housed in separate sheds. The birds were housed under negative pressure with filtered air supply. Group A was also vaccinated at 1 day old by oculonasal application with a live aMPV vaccine (RTCV1194; Intervet International, Boxmeer, The Netherlands), based on a subtype B chicken isolate (Cook *et al*., 1995). Group B received no further vaccinations.

During rear the males were culled as soon as they could be identified. At 16 weeks of age, 15 aMPV vaccinated birds (group A) were vaccinated intramuscularly with a multivalent inactivated vaccine containing Newcastle disease virus, EDS-76, infectious bronchitis (M41 & D274), infectious bursal disease virus and aMPV antigens (Intervet International). This group was designated group I (Table 1). Group B was divided into three groups, groups II, III and IV. Each group was housed in a different shed and birds were housed in individual laying cages. Protective clothing was used in every shed and routine biosecurity procedures were followed (sampling of non-challenged birds prior to challenged groups). At peak of lay (27 weeks of age) groups I and II were challenged intravenously into the wing vein, and group III oculonasally with the virulent aMPV isolate. Each bird received $\log_{10}$ 6.17 median ciliostatic doses of virus in 1 ml. Group IV was kept as uninfected controls.

Swabbing of birds

After challenge 15 tagged birds in each group were swabbed in duplicate from the pharynx and the cloaca using commercial cotton wool swabs at intervals from 2 to 28 days post challenge.

Table 1. *Experimental design used to study the effect of intravenous or oculonasal avian metapneumovirus challenge in vaccinated and non-vaccinated laying hens*

Group	Live priming at day-old	Inactivated vaccine (16 weeks)	Intravenous challenge (at peak of lay)	Oculonasal challenge (at peak of lay)
I	+[a]	+	+	−
II	−[b]	−	+	−
III	−	−	−	+
IV	−	−	−	−

[a] Vaccinated/challenged.
[b] Not vaccinated/not challenged.

At each sampling date cloacal and pharyngeal swabs of the 15 birds in groups I to IV were taken in groups of five and pooled. This made up three subgroups, C1 to C3, for the cloacal swabs and three subgroups, P1 to P3, for the pharyngeal swabs per group I to IV. The subgroup assignment for each bird was kept throughout the whole experiment, which means that the swabs from the same five birds were always pooled for further processing.

For live virus isolation each set of five swabs was placed in a labelled universal bottle containing 2.5 ml Eagle's minimal essential medium without serum as described earlier (Catelli *et al.*, 1998). Each universal bottle containing swabs was shaken vigorously and the swabs removed leaving as much fluid behind as possible. The centrifuged swab fluid was then transferred to a labelled cryotube and stored at -50°C. For PCR examination each set of five swabs was placed straight into a labelled tube. Each set of swabs was then air dried, returned to the tube and stored frozen at -50°C.

Virus isolation

Each swab fluid was passaged three times in chicken tracheal organ cultures. Passages 1 and 2 were blind passages. Then 0.1 ml was inoculated directly into each of five tubes, each tube containing two to four tracheal rings in 0.5 ml Eagle's serum-free minimal essential medium. These were harvested and pooled 3 days post inoculation and frozen at -50°C until passaged again.

For passage 3, 0.1 ml fluid harvested from passage 2 was inoculated into each of five tubes each containing one tracheal ring from which the medium has been removed. The tubes were incubated for 1 h at 37°C to allow virus absorption, and then each tube was overlaid with 0.5 ml medium warmed to 37°C. The cultures were examined at 7 and 10 days post inoculation for cilia activity as described by Catelli *et al.* (1998).

RNA detection

PCR was performed based on primers that hybridize to the G-gene, using a recently published method with some alterations (Hess *et al*., 2000). The five swabs taken from one subgroup were processed together for RNA isolation. RNA was isolated using the RNAeasy extraction kit (Qiagen, Hilden, Germany). Dried swabs were soaked in 500 µl RLT buffer containing 10 µl/1 ml mercaptoethanol. The description given by the manufacturer for this kit was followed throughout the whole protocol until the final washing step. This was performed twice using 25 µl RNAse-free water each time. From the eluted material 4.5 µl RNA template were used for cDNA transcription with the Omniscript kit (Qiagen) in a final volume of 20 µl according to the manufacturer's instructions. For the RT reaction 50 pmol of a slightly modified $G3^{++}$ primer (5′-gggacaagtatctctatggggtc-3′) published by Juhasz & Easton (1994) was used. The mixture was incubated for 1 h at 37°C and enzyme inactivation was performed at 93°C for 5 min. The single RT-PCR was performed using the aforementioned forward primer $G3^{++}$ together with the reverse primer 446^{-} (Bäyon-Auboyer *et al*., 1999) and 4 µl cDNA. After an initial denaturation step at 95°C for 5 min, 35 cycles with the following cycling programme were performed: 95°C for 1 min, 60°C for 1 min, 72°C for 1.5 min. Five microlitres of the PCR mixture were analysed on a 0.5% agarose gel. For nested RT-PCR the same conditions were used as already described for the single RT-PCR except that only 25 cycles of the first PCR were performed. After that, 2 µl

PCR reaction were transferred into the second round of amplification for another 30 cycles. A reverse primer X^- (5′-acaatcaaaaccacccagtaca-3′) and a forward primer B^+ (5′-gagccaaaaaatactgttaggact-3′) were used for further amplification. The whole amplification mixture of the second PCR was analysed on a 0.5% agarose gel.

Results

Clinical signs

Severe clinical signs, with birds being lethargic and looking sick, were found mostly between 7 and 10 days post challenge (d.p.c.) in the non-vaccinated, intravenous challenge group II. Coughing started as early as 5 d.p.c. and persisted until 13 d.p.c. Up to five birds with diarrhoea were noticed in the intravenously challenged group II during the first 14 d.p.c. In the oculonasal challenge group III one bird was lethargic and appeared sick 6 d.p.c. In comparison, no severe clinical signs were found in the vaccinated intravenous challenge group I or the control group IV.

Concurrent with severe clinical signs, a drop in egg production occurred in the non-vaccinated intravenously challenged group II. One week post challenge, egg production dropped from 85.7% to 56.2%. There was no drop in egg production in the vaccinated and intravenously challenged or in the non-vaccinated oculonasally challenged groups (groups I and III). In fact, the highest egg production, 93.3%, was recorded from the vaccinated, intravenous challenge group I at the time of the drop in egg production seen in group II.

Virus isolation

The results of virus isolation are presented in Table 2. No live virus was isolated from the vaccinated intravenous challenge group I or the control group IV. Live virus excretion was found in both non-vaccinated challenge groups. Virus was isolated from all pharyngeal swab fluids from challenge group III 2 and 5 d.p.c. No virus was isolated from cloacal swab fluids. No virus was isolated at 7 or 12 d.p.c (no samples taken at later times were examined for live virus).

Most virus-positive samples were found in the intravenous challenge group II. Virus was isolated from one pharyngeal swab fluid at 2 d.p.c. and all pharyngeal swab fluid taken at 5 and 7 d.p.c. Virus was also isolated from cloacal swab fluids C2 and C1 taken at 5 and 7 d.p.c., respectively.

Table 2. *Comparison of virus isolation, RT-PCR and nested RT-PCR to detect viable aMPV and/or viral RNA*

Group	Subgroup[a]	2 d.p.c.	5 d.p.c.	7 d.p.c.	12 d.p.c.	16 d.p.c.	21 d.p.c.	28 d.p.c.
I, vaccinated, challenged intravenously	P1							
	C1			N[c]				
	P2	S[b]			N			
	C2			N	N			
	P3			N	N			
	C3							
II, non-vaccinated, challenged intravenously	P1		V[d]	V/S/N	N	N	S/N	N
	C1			V/S/N	N	N	N	N
	P2		V	V/S/N	N	N	N	
	C2		V	S/N	N	N	N	N
	P3	V	V	V/N	N			
	C3			N		N		
III, non-vaccinated, challenged oculonasally	P1	V	V	N	N	N	N	N
	C1			N	N	N	N	N
	P2	V	V	N	N	N	N	N
	C2			N	N		N	
	P3	V	V	N/S	N	N	N	
	C3			N	N	N	N	N
IV, non-vaccinated, no challenge	P1							
	C1							
	P2							
	C2							
	P3							
	C3							

All swabs from groups I to IV taken between 2 and 12 d.p.c. were examined for virus isolation. All swabs taken from groups I to III between 2 and 28 d.p.c. were investigated by single RT-PCR, together with the swabs taken from group IV at 7 d.p.c.. All swabs taken from groups II and III between 7 and 28 d.p.c., from group I collected at 7 to 16 d.p.c. and those from the birds in group IV taken at 7 d.p.c were processed by nested RT-PCR.

[a] Each subgroup consists of five birds from which pharyngeal (P) and cloacal (C) swabs were taken and pooled for further processing as a single sample.

[b] Positive by single RT-PCR.

[c] Positive by nested RT-PCR.

[d] Positive by virus isolation.

Single RT-PCR

The largest number of positive samples were found by single RT-PCR in group II at 7 d.p.c. (Table 2). Both pharynx and cloaca swab subgroups P1/C1 and P2/C2 were positive at this time. One pharynx sample (P1) was found positive at 21 d.p.c.. Only one positive sample P3 was found at 7 d.p.c. in group III; however, the samples prior to this time were not tested. A PCR fragment was amplified from sample P2 (group I) taken at 2 d.p.c. All other samples investigated from these groups stayed negative. None of the pooled swabs taken from group IV at 7 d.p.c. gave a positive signal.

Nested RT-PCR

The results for the nested RT-PCR are also presented in Table 2. As positive samples with single RT-PCR were mainly found 7 d.p.c., this time point was used as the starting point to investigate swabs by nested RT-PCR. No viral RNA was detected from the swabs from control group IV. Positive results were found in the challenged groups I to III.

The majority of swabs taken from groups II and III were positive until the experiment was terminated at 28 d.p.c.. For group III the results obtained at two different sampling times 9 days apart from each other are given in Figure 1a. All tested swabs were found positive at the first sampling date whereas some negative results were found later on. The whole PCR material was separated on the gel showing somewhat weaker signals at the second sampling date. In group I, positive results were only found up to 12 d.p.c. giving rather weak signals (Figure 1b).

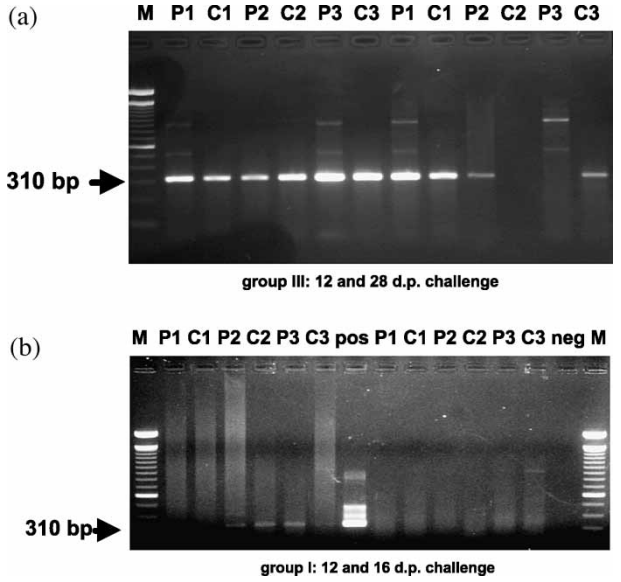

Figure 1. *Electrophoresis of the nested RT-PCR products. Amplification products from the swabs taken: (1a) from non-vaccinated group III at 12 and 28 d.p.c. challenge with aMPV oculonasally, and (1b) from vaccinated group I at 12 and 16 d.p.c. challenge with aMPV intravenously. neg, negative reagent control; pos, positive control (RNA from aMPV (RTCV 1194 subtype B)); M, DNA size marker (100 base pair DNA ladder, Invitrogen).*

Discussion

In recent years there has been some empirical field evidence that aMPV is involved in laying problems in chickens, similar to that described in turkeys. However, there are only a few experimental studies dealing with this subject. The objective of the present study was to obtain further data on the excretion and detection of aMPV in laying chickens by challenging experimentally vaccinated and non-vaccinated hens, either intravenously or oculonasally at peak of lay.

Using the subtype B chicken isolate and the intravenous challenge as described by Cook *et al.* (2000), it was again possible to induce a drop in egg production and severe clinical signs in layers. These results are contrary to those reported by Majó *et al.* (1995) and Khehra & Jones (1999), who also performed *in vivo* studies. The most obvious explanation for this discrepancy could be the use of different challenge viruses, as already discussed by Cook *et al.* (2000).

In the present study, attention was concentrated on detection of live virus or virus antigen excretion from the respiratory and intestinal tract. Live virus could only be isolated for 7 days post intravenous challenge and 5 days post oculonasal challenge. This agrees with several earlier studies. Catelli *et al.* (1998) reported the isolation of aMPV in young chickens for 5 d.p.i. and Van de Zande *et al.* (1999) found the same result with two different subtypes in turkeys. Virus isolation after 5 d.p.i. is only possible if the infection is in the lungs and air sacs (Cook *et al.,* 1993). The short-term live virus excretion after intravenous or oculonasal challenge, and the fact that virus excretion precedes the clinical signs is typical of aMPVs (Cook, 2000). Based on the present study this observation is now confirmed for laying chickens.

If viable virus excretion via the faeces is taken as the sole criteria for infection of the reproductive tract then no viraemia would have occurred in the present experiment after oculonasal infection. Live virus was isolated from two different cloacal swab subgroups (group II, C1 and C2) from the intravenous challenge group. In this context it is worth noting that for one subgroup of five birds (P2/C2) viable virus excretion via the respiratory and the faeces occurred at the same time, 5 d.p.c.. A virus isolated from cloacal swabs can originate from the digestive or the reproductive tract. It could be speculated whether the viable virus isolated from C1 and C2 of group II originated from the reproductive tract. Most probably it does, as no viable virus was detected in the faeces of the birds challenged oculonasally (group III), even though

all subgroups tested at 2 and 5 d.p.c. excreted virus by the respiratory tract. In agreement with this, Catelli *et al*. (1998) demonstrated in young chickens that several non-respiratory tissues including duodenum are not susceptible to aMPV, leaving two target sites in laying chickens for replication after intravenous challenge. However, many more positive swabs came from the respiratory tract, indicating a clear preference of aMPV for this site, especially in those birds challenged oculonasally.

The detection of viral RNA by single RT-PCR was as sensitive as virus isolation. Nested RT-PCR was more sensitive, with the majority of swabs taken from non-vaccinated birds found positive up to 4 weeks post challenge at which time the study was terminated, even though the amount of viral RNA detected by PCR was obviously declining throughout the experiment (Figure 1a). So far, studies in chickens using swab samples from the field to confirm the presence of aMPV RNA were mostly performed with nested RT-PCR, which is recommended to increase sensitivity (Cavanagh *et al*., 1999; Hess *et al*., 2000). Cook & Cavanagh (2002) mentioned in their technical review on aMPV detection that single RT-PCR sometimes fails to detect virus, whereas nested RT-PCR gave positive signals. Cavanagh *et al.* (1999) used degenerate primers to demonstrate in a semi-quantitative approach that levels of viral RNA excretion vary between different samples, underlining the importance of a sensitive detection system. In the present investigation only one forward primer was used in the second step of the nested RT-PCR, as the birds were infected with a well-defined subtype B isolate. Using the same PCR, with the addition of two specific primers in the second round of amplification, Wenzel & Hafez (2002) detected subtype A viral RNA from the respiratory tract of turkeys up to 4 weeks after vaccination and mixed infections with two subtypes were diagnosed after 5 weeks.

The effect of vaccination on live virus excretion and RNA detection was obvious. No live virus was detected from either the pharynx or the cloaca of intravenously challenged and vaccinated birds, and viral RNA could only be detected for a short time. Patnayak *et al.* (2002) performed studies in young chickens with the aMPV subtype C virus, present in North America. Using single RT-PCR, viral RNA could only be detected from non-vaccinated challenged birds, vaccinated birds being negative.

According to this and earlier experiments by Cook *et al.* (2000) it can be concluded that aMPV has at least two target sites for replication in laying hens after intravenous application, the respiratory and the reproductive tract. As well as new data on the persistence and detection, of aMPV, the present study shows that vaccination prevents virus excretion in laying chickens very efficiently. Since live virus can only be isolated for a short time in field cases in which aMPV may be involved in laying problems, the nested PCR could be a very helpful tool to confirm this presumption.

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RÉSUMÉ

Excrétion de Métapneumovirus aviaire chez des pondeuses exemptes d'organismes pathogènes spécifiés, vaccinées ou non

Des pondeuses leghorn blanches exemptes d'organismes pathogènes spécifiés vaccinées ou non ont été éprouvées au pic de ponte par voie intraveineuse ou oculonasale avec une souche de métapneumovirus aviaire (aMPV) de sous-type B, isolée de poulet. Les pondeuses non vaccinées éprouvées par voie intraveineuse ont présenté des signes cliniques sévères et une chute de ponte, alors que les pondeuses vaccinées n'ont pas été affectées. L'excrétion du virus a été mise en évidence dans les fecès et le tractus respiratoire des pondeuses non vaccinées jusqu'au 7ème jour après l'épreuve intraveineuse. Après l'épreuve oculonasale, l'excrétion du virus n'a pu être détectée qu'au niveau du tractus respiratoire durant 5 jours. L'excrétion du virus n'a pas été mise en évidence dans les fèces ni dans le tractus respiratoire des animaux vaccinés. Parallèlement à l'isolement du virus, la présence de l'ARN viral a été démontrée par RT-PCR. La RT-PCR nichée a été plus sensible et l'ARN viral a été détecté chez les pondeuses non vaccinées jusqu'au 28ème jour après les épreuves intraveineuse ou oculonasale, fin de l'expérience. L'ARN viral a été mis en évidence jusqu'au 12ème jour chez les pondeuses vaccinées. C'est la première étude qui a recherché l'excrétion de l'aMPV et l'ARN viral chez des pondeuses éprouvées, vaccinées ou non, dans les conditions expérimentales. Les résultats présentent de l'intérêt du point de vue de la persistance de l'aMPV et de la méthode de détection appropriée chez les pondeuses.

ZUSAMMENFASSUNG

Exkretion von aviärem Metapneumovirus (aMPV) in vakzinierten und nicht-vakzinierten spezifiziert pathogenfreien Legehennen

Vakzinierte und nicht vakzinierte spezifiziert pathogenfreie weiße Leghorn-Legehennen wurden auf dem Legeleistungshöhepunkt mittels intravenöser oder okulonasaler Inokulation mit einem virulenten Subtyp B-Hühnerstammm des aviären Metapneumovirus (aMPV) belastungsinfiziert. In den nicht-vakzinierten, intravenös infizierten Tieren wurden hochgradige klinische Symptome und ein Rückgang der Legeleistung induziert, während die vakzinierten Tiere nicht betroffen waren. Im Fäzes und Respirationstrakt der nicht vakzinierten Hennen wurde bis zu 7 Tage nach dem intravenösen Challenge eine Ausscheidung von Lebendvirus nachgewiesen. Nach der okulonasalen Belastungsinfektion konnte eine Virusexkretion nur im Respirationstrakt 5 Tage lang festgestellt werden. Bei den vakzinierten Tieren wurde weder im Fäzes noch im Respirationstrakt eine Lebendvirusausscheidung gefunden. Parallel zur Lebendvirusisolierung wurde die Präsenz von viraler RNS durch eine Einzel-RT-PCR nachgewiesen. Die nested RT-PCR war sensitiver, da virale RNS in den nicht-vakzinierten Tieren sowohl nach der intravenösen als auch nach der okulonasalen Belastungsinfektion bis zum 28. Tag, an dem das Experiment beendet wurde, entdeckt wurde. Bei den vakzinierten Tieren wurde virale RNS 12 Tage lang gefunden. Dies ist die erste Studie, die die Ausscheidung von aMPV und viraler RNS in vakzinierten und nicht-vakzinierten Legehennen nach experimenteller Belastungsinfektion untersucht. Die Ergebnisse sind von Bedeutung hinsichtlich der Persistenz von aMPV sowie der geeigneten diagnostischen Nachweismethode in Legehennen.

RESUMEN

Excreción de Metapneumovirus (aMPV) en ponedoras libres de patógenos específicos vacunadas y no vacunadas

Se inocularon experimentalmente en el pico de puesta vía intravenosa o oculonasal con una cepa virulenta de metapneumovirus aviar (aMPV) subtipo B cepa de pollo, ponedoras ligeras libres de patógenos específicos vacunadas y no vacunadas. Se reprodujeron signos clínicos graves y una caída en la puesta en las aves inoculadas no vacunadas mientras que las vacunadas no se vieron afectadas. Se demostró la excreción de virus vivo en las heces y el tracto respiratorio de las gallinas no vacunadas hasta 7 días post inoculación intravenosa. Tras la inoculación oculonasal la excreción viral sólo pudo detectarse en el tracto respiratorio hasta los 5 días. No se detectó excreción de virus vivo ni en las heces ni tracto respiratorio de las aves vacunadas. Al mismo tiempo que el aislamiento vírico, se demostró la presencia de ARN viral mediante RT-PCR simple. La RT-PCR anidada fue más sensible y el ARN viral pudo detectarse en aves no vacunadas hasta los 28 días post inoculación, tanto intravenosa como oculonasal, momento en que el experimento finalizó. El ARN viral se detectó hasta los 12 días en las aves vacunadas. Este es el primer estudio que investiga la excreción de aMPV y ARN viral en gallinas ponedoras vacunadas y no vacunadas inoculadas bajo condiciones experimentales. Los resultados son importantes en cuanto a la persistencia de aMPV y a la elección de un método de detección apropiado para el diagnóstico en ponedoras.