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Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain

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To improve the detection and molecular identification of infectious bronchitis virus (avian coronavirus), two reverse transcriptase-polymerase chain reaction (PCR) assays were developed. As 'diagnostic PCR', a set of consensus nested primers was selected from highly conserved stretches of the nucleocapsid (N) gene. As 'phylogeny' PCR, a fragment of the spike protein gene (S1) was amplified and the PCR products were directly sequenced. To study the phylogenetic relationships of the viruses from various outbreaks, studies of molecular epizootiology were performed in Sweden, a Nordic region, where the occurrence of natural cases of the disease is relatively low and the occasional use of live vaccine(s) is well recorded and monitored. The disease appeared in the region in 1994, associated with production problems among layers of various ages. During outbreaks in 1995 and 1997, both layers and broilers were affected. To reduce losses, a live attenuated vaccine has been applied since 1997. By examining 12 cases between 1994 and 1998, molecular epizootiology revealed that, before 1997, the viruses had gene sequences very similar to strains of the Massachusetts serotype. However, comparative sequence analysis of the S1 gene revealed that the identity was not 100% to any of the strains of this serotype that we analysed. A virus related to the Dutch-type strain, D274, was also identified on one farm. Surprisingly, from 1997, the year that vaccination commenced with a live Massachusetts serotype vaccine, the majority of viruses detected had S1 sequences identical to the live Massachusetts vaccine strain. This genetic relation to the vaccine virus was also confirmed by N gene sequence analysis. The studies of molecular epizootiology reveal a strong probability that the vaccination had lead to the spread of the vaccine virus, causing various disease manifestations and a confusing epizootiological situation in the poultry population.

Introduction

Infectious bronchitis virus (IBV) is a highly infectious and contagious pathogen of chickens worldwide. The genome of IBV, a member of the family *Coronaviridae*, contains a single-stranded, positive-sense RNA of 27.6 kb (Boursnell *et al.*, 1987). The virion is an enveloped pleomorphic particle approximately 80 to 100 nm in diameter and has three major virus-encoded structural proteins, which include the spike (S) glycoprotein, the membrane (M) protein, and the nucleocapsid (N) protein. The spikes of IBV are formed by post-translational cleavage of two separate polypeptide components designated S1 and S2 (Cavanagh, 1983a,b). The molecular identification of IBV is based mainly on analysis of the S1 protein gene (Lin *et al.*, 1991; Handberg *et al.*, 1999; Kingsham *et al.*, 2000; de

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Wit, 2000; Cavanagh, 2001a,b; Li & Yang, 2001). The N protein is closely associated with the RNA genome (Lai & Cavanagh, 1997).

IBV replicates in the respiratory and urogenital tract, causing various disease manifestations, and affecting both production and egg quality (King & Cavanagh, 1991). Since the first description by Schalk & Hawn (1931) in the US, many strains, which vary widely in virulence, have been identified (Fabricant, 1998). Serological studies revealed that IBV could be divided into more than 20 serotypes.

The control of infectious bronchitis (IB) is based on various policies of epizootiology, including the use of live attenuated vaccines. The live vaccines prepared from Massachusetts serotype strains provide good immunity against the homologous serotype. Thus, such vaccines have been used worldwide for decades (Sharma, 1999). However, the disadvantage of the live vaccine strains is that they are spreading in the field (Meulemans *et al.*, 2001). This creates an epizootiological problem, since the exact discrimination between live Massachusetts vaccine strains and Massachusetts wild strains is very difficult. A further problem is that immune response induced by a Massachusetts-type vaccine is often not sufficiently effective against new serotypes (Cavanagh *et al.*, 1997), which emerge due to changes in the IBV genome, including deletions, insertions, point mutations and, in some cases, recombinations (King, 1988; Wang *et al.*, 1994; Jia *et al.*, 1995; Lee & Jackwood, 2001). In such a confusing epizootiological situation, the rapid and accurate identification of newly emerging IBV variants is very important (King, 1988; Kwon *et al.*, 1993; Cavanagh *et al.*, 1999; Lee & Jackwood, 2001).

Considering these high risk factors, it is important to study the capacity of vaccine viruses to spread and initiate disease. Furthermore, reliable and practical methods are required to discriminate between wild viruses and spreading vaccine strains. To investigate this scenario, Sweden was chosen as the scene of our study. This is a region where the occurrence of natural cases of IB is relatively low and the occasional use of vaccine(s) is well recorded and monitored. In this country, the last natural outbreak of IB (before the present cases) occurred in the 1970s. No vaccination was practised before 1997.

Concerning natural cases of the present study, the first outbreak was recorded in February 1994, affecting layers, followed by second case in 1995 among layers and broilers, respectively. Since the losses became increasingly frequent, in spring 1997 the authorities permitted immunization against IBV using a live attenuated vaccine. After vaccination, production problems were observed in a number of flocks, and it could not be excluded that the vaccine virus was spreading and maintaining a very complicated epizootiological situation. These problems emphasized the need to: (i) develop a highly sensitive and specific novel method to detect and identify IBV; and (ii) to determine the genetic relationship of the viruses in the field cases, with special regard to possible spread of the vaccine strain in poultry populations.

To meet these purposes, two reverse transcriptase-polymerase chain reaction (RT-PCR) assays were developed. To detect all possible variants with appropriate sensitivity, a nested 'diagnostic' PCR was constructed, targeting the most conservative regions of the N gene of IBV. To perform further strain identification and phylogeny, a 'phylogeny' PCR assay was applied to amplify the most variable region of the S1 gene. The nucleotide sequences of the PCR products were determined and comparative analysis was performed to estimate the phylogenetic relationship of the viruses, with specific regard to a supposed spread and possible damage caused by the vaccine virus in the flocks.

Materials and Methods

Natural cases

Before 1997 no IB vaccines were applied in Sweden. Twelve cases, occurring on different farms between 1994 and 1998 (Table 1), were previously diagnosed as IB, using a blocking enzyme-linked immunosorbent assay (ELISA) test (L.H.M. Renström, unpublished results). The first outbreaks affected layers, causing production problems in 1994. The outbreaks in 1995 and 1997 happened among layers and broilers, respectively. The manifestations were drops in egg production, poor egg quality, coughing and sneezing. Virus isolation was not performed; the specimens from these 12 cases (Table 1) were analysed directly by PCR.

Table 1. *Viruses of the study*

Virus	Outbreak
SE/1096/97[a]	Layer 97–98
SE/1097/97	Layer 97–98
SE/1106/97	Layer 97–98
SE/1120/97	Layer 97–98
SE/50/98	Layer 97–98
SE/422/97	Layer 97–98
SE/423/97	Layer 97–98
SE/397/95	Scania 95
SE/722/95	Halland 95
SE/340/94[b]	Layer 94
SE/242/95[b]	Scania 95
SE/748/95	Halland 95
X[c]	Vaccine

[a] Named as suggested by Cavanagh (2001a): country/isolate number/year of isolation. SE, Sweden.

[b] In the case of these samples, the 'phylogeny' PCR (S1) was unsuccessful.

[c] Due to ethical reasons, the name of the vaccine strain is not given here.

Organ specimens

Specimens of tracheas and intestines were analysed. The samples were maintained separately.

Isolation of RNA

The organ specimens from the infected animals were homogenized, 1 g tissue in 8 ml phosphate-buffered saline without calcium and magnesium (PBS). Total RNA was prepared by mixing 166 µl homogenized organ, 245 µl PBS, 83 µl 6 × proteinase K buffer (0.01 M Tris (pH 7.8), 0.005 M ethylenediamine tetraacetic acid and 0.5% sodium dodecyl sulfate) and 5 µl proteinase K (14 mg/ml; Boehringer Mannheim, Germany). The mixture was incubated for 1 h at 55°C. The RNA was extracted by Trizol™ LS (Gibco) (Verhofstede *et al.*, 1996). The precipitated RNA was pelleted at 14 000 × *g* for 30 min, washed once with 400 µl of 70% ethanol, dried and dissolved in 50 µl freshly prepared diethyl pyrocarbonate-treated water (DEPC) (Fluka Chemi AG, Buchs, Switzerland), and used immediately or stored at −70°C (Vilcek *et al.*, 1994).

Synthesis of cDNA

Specimens of cDNA were synthesized in 25 µl reaction mixtures containing 5 µl specimen RNA, 5 µl DEPC-treated water and 1 µl random hexamers (0.02 U; Pharmacia Biotech, Uppsala, Sweden). RNA was denatured at 65°C for 5 min. Subsequently, the tubes were placed on ice, and 17 µl premix containing 1 µl RNAguard (24 U; Pharmacia), 2.5 µl each dNTP (2 mM; Pharmacia), 5 µl of 5 × first-strand reaction buffer (Gibco BRL; Bethesda, MD, USA), and 1 µl Moloney murine leukaemia virus reverse transcriptase (200 U; Gibco BRL) was added. The reaction mixtures were incubated at 37°C for 90 min followed by inactivation of the enzyme at 98°C for 5 min.

'Diagnostic' PCR

For diagnostic purposes, two primer pairs (N784 to N1145 and N791 to N1129, respectively) were designed to detect IBV in the animals. The primer sequences are situated in the nucleocapsid (N) gene. To ensure a very wide detection range, the primers were selected on the basis of a large collection of IBV sequences; all of the 14 nucleocapsid sequences available in GenBank (Table 2). Sequences, orientation and nucleocapsid positions of the primers are presented in Table 3. The amplification reaction was carried out in 50 µl mixtures containing 0.2 mM each deoxynucleotide, 10 pmol each primer (the external primers N984 and N1145), 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CO, USA), and 3 µl cDNA. Two droplets of mineral oil (Sigma) were added to prevent evaporation. Amplification was performed with a thermal profile of 94°C for 45 sec, 60°C for 1 min and 72°C for 2 min. This cycle profile was repeated 35 times with a final extension at 72°C for 7 min.

After the first run of amplification, 1 µl amounts of the PCR products were added to a fresh reaction tube containing identical reagents and the internal primers (N794 and N1129). The thermal profile of the second run of amplification was 94°C for 45 sec, 53°C for 1 min and 72°C for 1 min.

For visualization, 5 µl amounts of the PCR products were electrophoresed in 2% agarose gels. After electrophoresis, the gels were stained in ethidium bromide and viewed under ultraviolet light.

'Phylogeny' PCR and sequence analysis

As judged by comparative studies of 37 sequences available in GenBank (Table 4), the first half of the S1 gene allows a very high phylogenetic resolution. Accordingly, two primers were selected to amplify a region (840 bases) from the first half of the S1 gene. The thermal profile of the 'phylogeny' PCR was 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min. The PCR products were sequenced by an ABI PRISM device with dye terminators (Applied Biosystems Inc., Foster City, CA, USA). The nucleotide and deduced amino acid sequences were aligned with the aid of the multiple programs DNASTAR (DNASTAR Inc., Madison, WI, USA), using the Clustal method (Verhofstede *et al.*, 1996). In the alignment, all the sequences available from the S1 gene were included.

Accession numbers of sequence data

The nucleotide sequence data reported in this paper have been submitted to the GenBank sequence database and have been assigned the accession numbers AF420320 to AF420330.

Table 2. *The names and the accession numbers of the strains from GenBank used for design of diagnostic primers (N gene)*

Name	Accession number	Name	Accession number
Vic_S	U52594	Ark99	M85244
Cu-T2	U04805	Beaudette	M95169
Gray	M85245	M41	M28566
N1/62	U52596	N1/88	U52599
N2/75	U52598	N9/74	U52597
Q3/88	U52600	H52	AF352310
V18/91	U52601	V5/90	U52595

Table 3. *Sequence, orientation and nucleotide position of the primers*

Name	Sequence	Position
Diagnostic primers selected from the N gene		
N784	AAT TTT GGT GAT GAC AAG ATG A	763 to 784[a]
N1145	CAT TGT TCC TCT CCT CAT CTG	1145 to 1165
N791	GTG ATG ACA AGA TGA ATG AGG A	770 to 791
N1129	CAG CTG AGG TCA ATG CTT TAT C	1129 to 1150
Primers of phylogeny from the S1 gene		
CO1[c]	TGA CTC TTT TGT KTG CAC TAT	20390 to 20416[b]
CO2	AAA TTA TAA TAA CCA CTC TGA	21255 to 21278

[a] The numbers are indicative of the exact position of the primers on the N gene of Beaudette (accession number M95169) used as reference.
[b] The numbers are indicative of the exact position of the primers on the S1 gene of Beaudette (accession number M95169) used as reference.
[c] Oligonucleotide CO1 was generate at position 14 from the S^{-1} end: K=G+T.

Table 4. *The names and the accession numbers of the strains from GenBank used for design of phylogeny primers (S1 gene)*

Name	Accession number	Name	Accession number
DE/072	AF274435	SE 17	AF239984
JMK	AF239982	Florida 18288	AF027512
GAV-92	AF094817	D41	AF036937
CU-T2	U49858	CV-1686	AF027511
CV-9437	AF027510	CV-56b	AF027509
B1648	X87238	UK/918/67	X64737
M41	X04722	D274	X15832
N2/75	U29523	N1/62	U29522
V18/91	U29521	V5/90	U29520
UK/2/91	Z83976	Q3/88	U29451
N3/62	U29453	N9/74	U29452
N1/88	U29450	KB8523	M21515
H120	M21970	variant 2	AF093796
Gray	L18989	PP14	M99483
3668–4	AF095702	4/91 attenuated	AF095702
4/91 pathogenic	AF0937794	Ark DPI	AF006624
Ark99	L10384	Beaudette	X02342
UK/7/91	Z83975	1013	AF027508
Beaudette	M95169		

Results

'Diagnostic' PCR

Using primer pairs N784 to N1145 and N791 to N1129 from the highly conservative N gene in nested RT-PCR, IBV was detected in all the 12 tested clinical samples. By testing reference viruses Netherlands/D1466/78, Netherlands/H120/55, UK/7/91 and UK/918/68 (kindly provided by Dr D. Cavanagh), the primers gave positive reactions with all of these strains. In each of these cases, PCR products were detected in the first run of amplification. However, to provide the possible highest sensitivity of virus detection, we always used nested PCR throughout these studies.

'Phylogeny' PCR and sequence analysis

Except for the viruses SE/242/95 and SE/340/94, the S1 gene RT-PCR system detected the virus in all specimens collected between 1994 and 1998. The PCR products were suitable to determine the phylogenetic relations of the detected viruses, in comparison with sequences available in GenBank. Figure 1 shows the dendrogram prepared from the sequence alignments.

The phylogenetic tree demonstrates that viruses SE/748/95, SE/397/95, SE/1097/97, SE/1120/97, SE/1096/97, SE/1106/97, SE/422/97, SE/423/97 and SE/50/98 detected from the outbreaks at Halland 95, Scania 95 and Layer 97–98 (Table 1) belonged to the Massachusetts type. One early isolate (SE/722/95 from the Halland 95 outbreak) was shown in the part of the S1 gene that was examined to have 99% identity with virus D274, a serotype of IBV first isolated in the Netherlands (Davelaar *et al.*, 1984; Cavanagh *et al.*, 1992).

From 1997, the detected viruses showed a new profile. The S1 sequences of four viruses (SE/1097/97, SE/1096/97, SE/1120/97 and SE/50/98) were completely identical to those of the live vaccine strain used in the country (termed only X here for ethical reasons), while three other viruses (SE/1106/97, SE/422/97 and SE/423/97) showed in the selected part of the S1 gene an identity between 86 and 94% to X (Figure 1).

To confirm the results the S1 gene phylogeny, an N gene phylogenetic tree was also prepared (not shown). The N gene phylogenetic tree confirmed the genome identity between the SE/1096/97, SE/1097/97, SE/1120/97 and SE/50/98 samples and vaccine strain X (Kusters *et al.*, 1989; Williams *et al.*, 1992).

Discussion

The results of the diagnostic RT-PCR confirmed the highly conserved nature of the selected region of the N gene. Although already the first run of RT-PCR gave positive results with each sample and virus strain, we always applied a second run of amplification in a nested PCR, in order to provide the possible highest sensitivity of virus detection. A high number experiments has proven that, compared with single-run PCR, nested PCR assays are more sensitive (Belák & Ballagi-Pordány, 1991; Ballagi-Pordány *et al.*, 1992). Thus, we believe that this higher sensitivity is required for a safe diagnosis. On account of the broad reactivity of the primers chosen by the set of 14 nucleocapsid gene

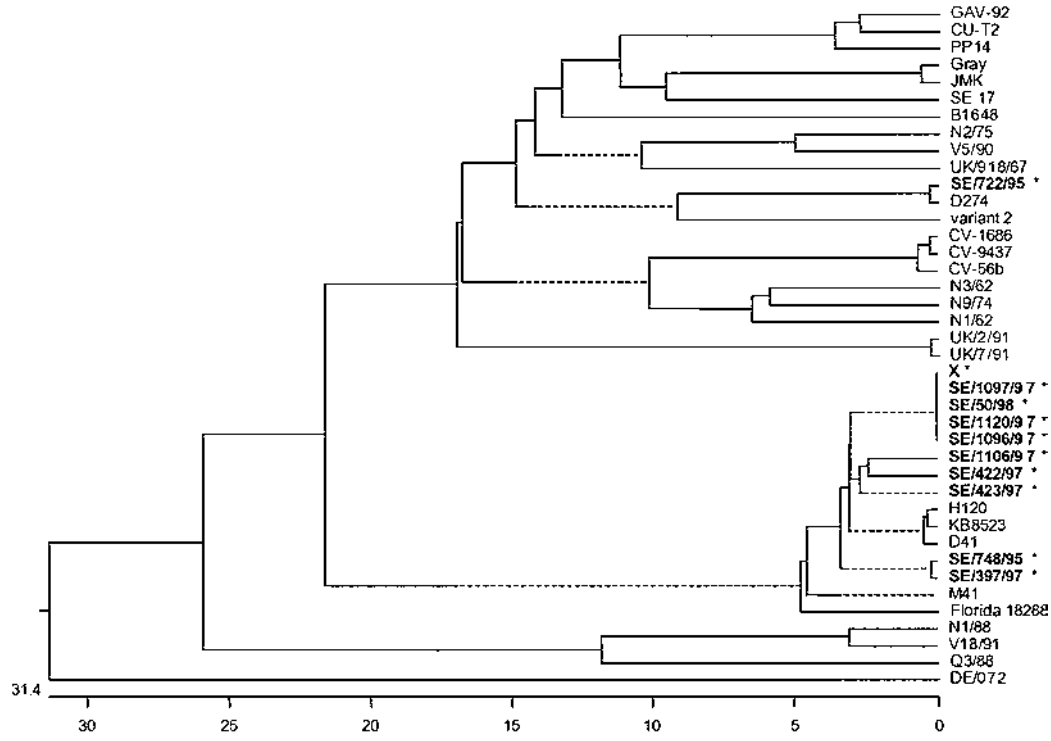

Figure 1. *Phylogenetic relationships of the Swedish IBV viruses based on S1 gene sequences compared with those in the databanks. The majority of the Swedish samples were clusterd with M41 and H120, typical representatives of the Massachusetts serotype. Sample SE/722/95 alone was shown in the selected part of the S1 gene to have 99% identity to Netherlands/D274/78 of the D274 serotype. The names of the viruses in the present study are marked with asterisks and shown in bold. The scale is indicative of the genetic distance.*

sequences available in GenBank, the developed PCR assay can be recommended for the general detection and identification of IBV variants in the field. The nested assay provides a powerful, novel tool in IB detection. One can argue that the highly sensitive nested PCR may yield false-positive results, and thus not be suitable for routine diagnosis. However, using all the precautions and safety arrangements of our routine diagnostic PCR laboratory (Belák & Ballagi-Pordány, 1993), carry-overs and cross-contaminations are practically eliminated and false-positive results are avoided. If necessary, the elimination of false-negative results is also possible by applying internal controls, like mimics, used in our other routine diagnostic PCR assays (Ballagi-Pord ány & Belák, 1996).

To develop a PCR assay for the phylogenetic study and to obtain results comparable with those of other authors, we have selected the first 5′ part of the S1 gene for amplification. The RT-PCR system of the S1 gene proved to be suitable for determining phylogenetic relationships of the field samples (Saif, 1993; Sapats *et al.*, 1996) except for two viruses, SE/242/95 and SE/340/94. The reasons why our 'phylogeny' RT-PCR assay failed to amplify these two viruses might either be an insufficient amount of the RNA or some mismatches might have been in the S1 gene of these viruses, compared with the sequences of the 'phylogeny' primers.

Similarly to the 'diagnostic' RT-PCR, no difference was revealed by 'phylogeny' RT-PCR in the results using RNA from the two different sources (trachea and intestine). For this reason, the further analysis was based entirely on the viral RNA obtained from the trachea samples. The present study revealed that viruses belonging to the Massachusetts serotype and a Dutch serotype caused natural cases in Sweden in the period 1994 to 1998. On most of the farms, Massachusetts-related strains were detected, but in one case the virus was 99% identical to strain D274. It is interesting that the D274-related strain (SE/722/95) was isolated in a farm situated not far (approximately 5 km) from another farm where a Massachusetts-related virus (SE/748/95) was detected about 2 months earlier. This means that various variants of the virus were circulating simultaneously and independently from each other in this small geographic region. The possible origin of virus SE/722/95 is unclear. It can be a wild-type relative of D274. However, since D274 virus was also used as a live vaccine, it cannot be excluded that virus SE/722/95 is a D274 vaccine derivative, imported into the country. However, considering the slight genetic differences and the lack of further data, these questions remain unanswered.

The further nine viruses showed a very interesting genetic divergence. The viruses detected in the early cases (before 1997) showed genetic patterns close to strains of the Massachusetts serotype. However, complete identity was not found in comparison with any of the tested strains

of this serotype. Obviously, these might represent natural cases of IB. The close genetic relationship to the Massachusetts serotype raises some questions again that vaccinated chickens were probably purchased from some broiler company abroad where a vaccine based on a Massachusetts strain was used. Considering, that no vaccination was practised in Sweden before 1997, one can speculate that such viruses may originate only from abroad. However, it is probable that the observed viruses are not of vaccine origin, but are natural cases of IB.

Surprisingly, after introduction of IB immunization in 1997, all detected viruses showed very strong genetic relatedness to the vaccine strain. By S1 sequence analysis, four viruses showed total identity and three further ones showed identity of between 86 and 94% to the strain used in the live attenuated vaccine. All these viruses were detected in unvaccinated flocks with production problems. One can consider three main possibilities: (i) natural cases again, the causative virus being closely related or identical to the vaccine strain with regard to S1 sequence; (ii) natural superinfection happened, the vaccine virus and the field virus of another serotype both being present, and only the Massachusetts-type vaccine strain being detected by the RT-PCR; and (iii) the vaccine strain was spreading to the unvaccinated flocks and caused the observed production problems.

Considering that the genetic variability of the S1 region is very high, the first scenario seems to be very unlikely. IBV can cause persistent infections, so the type of co-existence in possibility (ii) could happen (Capua *et al.*, 1999). Having no previous data about the causative virus, the 'phylogeny' primers were chosen to work on a broad set of IBV genotypes, not only the Massachusetts type strains. It seems to be unlikely that some other genotype of IBV could have escaped detection. These findings are supported by the ELISA results because, after turning positive, no rising titres were revealed that would have also indicated the fact of superinfection. However, the obtained data emphasize very strongly the third scenario, the spread of the vaccine virus. The nucleotide sequences of the viruses SE/1097/97, SE/1096/97, SE/1120/97 and SE/50/98 and the vaccine strain were totally identical in a stretch of 840 S1 nucleotides, strong evidence that the five viruses are identical. One could still argue against this identity, considering that recombination is a characteristic feature of IBV (Jia *et al.*, 1995; Kottier *et al.*, 1995; Wang *et al.*, 1997; Lee & Jackwood, 2000). It may be that a virus obtains a block of heterologous sequences from another strain in its S1 region and that this can disturb the exact classification of the serotypes and/or the strains (Jia *et al.*, 1995). This could be an explanation; that the four viruses have another origin, but not vaccinal. However, if so, why did the four viruses 'pick up' exactly the same 840 nucleotide sequences of the vaccine strain? In addition, what is the origin of these sequences, if not the vaccine itself?

Considering that the variability of S1 is very high not only for the recombinative events, but also for point mutations, insertions and also deletions, one can assume, if the entire 840 nucleotide long segment of S1 between the field viruses and a vaccine is identical, that the vaccine origin of the field viruses is the most probable explanation.

A further question is how should we consider the three further viruses (SE/1106/97, SE/422/97 and SE/423/97), which are not totally identical to the vaccine strain, but closely related in S1? One explanation is that these are also originating from the vaccine strain, but during passage in the field point mutations were accumulated in their S1 gene.

To avoid erroneous conclusions with regard to phylogeny, it is recommended to analyse the phylogenetic relations on more than one gene (Zwaagstra *et al.*, 1992). Therefore, phylogenetic studies were also performed in the N gene of IBV. These observations unequivocally confirmed the S1 phylogeny, indicating the vaccine origin of the discussed viruses.

In summary, sensitive novel approaches are presented here: (i) to detect rapidly all possible variants of IBV that can be warranted by the broad reactivity of the primers used in this study; and (ii) to identify genetic variants and to determine phylogeny. The N and S gene RT-PCRs may also be useful for detecting coronaviruses in other avian species; turkeys and pheasants are naturally infected by coronaviruses that are genetically very similar to IBVs (Breslin *et al.*, 1999a,b; Guy, 2000; Cavanagh, 2001a,b; Cavanagh *et al.*, 2001, 2002). Our study has revealed very strong evidence for the spread of a live virus vaccine strain in large populations of poultry (causing damage) and a confusing epizootiological situation. These findings should be considered during the practical work to combat IB in poultry populations.

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RÉSUMÉ

Epizootiologie moléculaire du virus de la bronchite infectieuse en Suède: implication d'une souche vaccinale.

Dans le but d'améliorer la détection et l'identification moléculaire du virus de la bronchite infectieuse (IBV, coronavirose aviaire), deux tests de transcription inverse et d'amplification en chaîne par polymérase ont été développés (RT-PCR). Pour les études diagnostiques par PCR, un jeu de sondes nichées a été sélectionné à partir de zone hautement conservée du gène de la nucléocapside (N). Pour les études de phylogènie par PCR, un fragment du gène de la protéine de la spicule (S1) a été amplifié et les produits PCR ont été directement séquencés. Dans le but d'étudier les relations phylogénétiques des virus de différents cas, des études d'épidémiologie moléculaire ont été réalisées en Suède, une région nordique où l'apparition de cas spontanés de maladie est relativement faible et l'utilisation occasionnelle de vaccin(s) vivant(s) est bien suivie.

La maladie est apparue dans la région en 1994, associée à des problèmes de production chez les pondeuses d'âges différents. Lors des cas enregistrés entre 1995 et 1997, les pondeuses et poulets de chair ont été affectés. Dans le but de réduire les pertes, un vaccin vivant atténué a été utilisé, à partir de 1997. L'examen en épizootiologie moléculaire de 12 virus isolés entre 1994 et 1998 révèle qu'avant 1997 les virus présentaient des séquences très similaires à celles des souches de sérotype Massachusetts. Cependant, l'analyse comparative des séquences du gène S1 a révélé que l'identité n'était pas de 100% pour toutes les souches de ce sérotype que nous avons analysées. Un virus proche de la souche hollandaise D274 a également été identifié dans un élevage. De façon surprenante, à partir de 1997, année du début de la vaccination avec un vaccin vivant de sérotype Massachusetts, la majorité des virus isolés ont présenté des séquences de S1 identiques à la souche vaccinale vivante Massachusetts. Cette relation génétique à un virus vaccinal a également été confirmée par l'analyse de la séquence du gène N. Les études d'épizootiologie moléculaire révèlent une forte probabilité que la vaccination a permis la diffusion d'un virus vaccinal, causant différentes manifestations de maladie et brouillant la situation épizootiologique de la population avicole.

ZUSAMMENFASSUNG

Molekulare Epizootiologie des aviären Bronchitisvirus in Schweden, die auf die Beteiligung eines Vakzinestammes schließen lässt

Um den Nachweis und die molekulare Identifizierung von Bronchitisvirus (IBV, aviäres Coronavirus) zu verbessern, wurden zwei Reverse-Transkriptase-Polymerase-Kettenreaktion (RT-PCR)-Tests entwickelt. Für eine "diagnostische PCR" wurde ein Set von verschachtelten Primern aus hoch konservierten Abschnitten des Nukleokapsid (N)-Gens ausgewählt. Für eine "Phylogenese"-PCR wurde ein Fragment des Spikeprotein-Gens (S1) amplifiziert und die PCR-Produkte wurden unmittelbar sequenziert. Um die phylogenetischen Beziehungen der Virusstämme von verschiedenen Ausbrüchen zu untersuchen, wurden Untersuchungen der molekularen Epizootiologie in Schweden, einer nordischen Region, durchgeführt, wo das Vorkommen von natürlichen Fällen der Krankheit relativ selten ist und die gelegentliche Verwendung von Lebendvakzine(n) gut dokumentiert und überwacht wird.

Die Krankheit tauchte 1994 in der Region auf und war mit Produktionsproblemen unter den Legehennen verschiedener Altersgruppen verbunden. Während der Ausbrüche in den Jahren 1995 und 1997 waren sowohl Legehennen als auch Broiler erkrankt. Um die Verluste zu reduzieren, wird seit 1997 eine attenuierte Lebendvakzine eingesetzt. Bei der Untersuchung von 12 Fällen zwischen 1994 und 1998 zeigte die molekulare Epizootiologie, dass die Viren vor 1997 Gen-Sequenzen hatten, die denen von Stämmen des Serotyps Massachusetts sehr ähnlich waren. Die vergleichende Sequenzanalyse des S1-Gens zeigte jedoch, dass die Identität mit keinem der von uns analysierten Stämme dieses Serotyps 100% betrug. Auch ein mit dem holländischen Referenzstamm D274 verwandtes Virus wurde auf einer Farm identifiziert. Seit 1997, dem Jahr, in dem die Impfung mit einer Lebendvakzine vom Serotyp Massachusetts begann, hatte überraschenderweise die Mehrzahl der nachgewiesenen Viren S1-Sequenzen, die mit der des Massachusetts-Vakzinestammes identisch waren. Diese genetische Beziehung zu dem Vakzinevirus wurde außerdem durch die N-Gen-Sequenzanalyse bestätigt. Die Untersuchungen der molekularen Epizootiologie offenbaren eine hohe Wahrscheinlichkeit dafür, dass die Vakzinierung zu einer Ausbreitung des Impfvirus geführt hatte, wodurch verschiedene Krankheitsmanifestationen und eine verwirrende epizootiologische Situation in der Geflügelpopulation verursacht wurden.

RESUMEN

Epizootiología molecular del virus de bronquitis infecciosa en Suecia que indica la participación de una cepa vacunal

Con el fin de mejorar la detección y la identificación molecular de virus de bronquitis infecciosa aviar (IBV, coronavirus aviar), se desarrollaron dos métodos de transcriptasa reversa-reacción en cadena de la polimerasa (RT-PCR). Como "PCR de diagnóstico" se desarrollaron un grupo de cebadores anidados consensuados a partir de secuencias muy conservadas del gen de la nucleocápside (N). Como PCR de "filogenia", se amplificó un fragmento del gen de la proteína *spike* (S1) y los productos de PCR se secuenciaron directamente. Con el fin de estudiar las relaciones filogenéticas de los virus de varios brotes epidémicos, se realizaron estudios de epizootiología molecular en Suecia, una región nórdica, donde hay un bajo nivel de casos naturales de esta enfermedad y donde el uso de vacuna(s) está bien controlado y monitorizado.

La enfermedad apareció en la región en 1994, asociada a problemas de producción en ponedoras de diferentes edades. Durante las epidemias de 1995 y 1997, se vieron afectados tanto los pollos de engorde como las gallinas ponedoras. Para reducir las pérdidas, se ha administrado una vacuna viva atenuada desde 1997. Mediante el examen de 12 casos entre 1994 y 1998, la epizootiología molecular reveló que antes de 1997 los virus tenían secuencias génicas muy similares a las cepas del serotipo Massachusetts. Pero, al analizar comparativamente las secuencias del gen S1, se observó que ninguna de las cepas de este serotipo analizada presentaba una identidad del 100%. Un virus relacionado con la cepa de tipo holandés, D274, fue identificada en una de las granjas. Sorprendentemente, desde 1997, el año en el cual empezó la vacunación con una vacuna viva del serotipo Massachusetts, la mayoría de los virus detectados presentaron una secuencia de la S1 idéntica a la de la vacuna viva de la cepa Massachusetts. Esta relación genética con el virus vacunal fue también confirmada por análisis de la secuencia del gen N. Los estudios de epizootiología molecular revelaron que con bastante probabilidad la vacunación conllevó la diseminación del virus vacunal, causando varias manisfestaciones de la enfermedad y una confusa situación epizootiológica en la población avícola.