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Host Intraspatial Selection of Infectious Bronchitis Virus Populations

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SUMMARY. Arkansas (Ark)-type infectious bronchitis virus (IBV) subpopulations with an S gene sequence distinct from the vaccine predominant consensus were previously found in the upper respiratory tract of chickens within 3 days after inoculation. This finding indicated that a distinct virus subpopulation was rapidly positively selected by the chicken upper respiratory tract. We hypothesized that during host invasion, the replicating IBV population further changes as it confronts the distinct environments of different tissues, leading to selection of the most fit population. We inoculated 15-day-old chickens with 10^4 50% embryo infective doses of an Ark-type IBV commercial vaccine via the ocular and nasal routes and characterized the sequences of the S1 gene of IBV contained in tear fluid, trachea, and reproductive tract of individual chickens at different times postinoculation. The predominant IBV phenotype contained in the vaccine (before inoculation) became a minor or nondetectable population at all times in all tissues after replication in the majority of the chickens, corroborating our previous findings. Five new predominant populations designated component (C) 1 through C5, showing distinct nonsynonymous changes, i.e., nucleotide changes resulting in different amino acids encoded and thus in a phenotypic change of the predominant virus population, were detected in the tissues or fluids of individual vaccinated chickens. Due to the different biochemical properties of some amino acids that changed in the S1 glycoprotein, we anticipate that phenotypic shift occurred during the invasion process. Significant differences were detected in the incidence of some distinct IBV predominant populations in tissues and fluids; e.g., phenotype C1 showed the highest incidence in the reproductive tract of the chickens, achieving a significant difference versus its incidence in the trachea ($P < 0.05$). These results indicate for the first time that IBV undergoes intraspatial variation during host invasion, i.e., the dominant genotype/phenotype further changes during host invasion as the microenvironment of distinct tissues exerts selective pressure on the replicating virus population.

RESUMEN. Selección intraespacial por el hospedador sobre las poblaciones del virus de la bronquitis infecciosa.

Subpoblaciones del virus de la bronquitis infecciosa serotipo Arkansas con secuencias del gen S que son distintas a la secuencia consensuada predominante del virus vacunal, han sido encontradas previamente en el tracto respiratorio superior de pollos dentro de los tres días después de la inoculación. Este hallazgo indica que una subpoblación viral diferente fue seleccionada rápidamente de manera positiva por el tracto respiratorio superior del pollo. Se estableció la hipótesis de que durante la invasión al hospedador, la población del virus de la bronquitis infecciosa que se está replicando se modifica de acuerdo con los ambientes diferentes a los que se va enfrentando en los distintos tejidos, con la tendencia a seleccionar la población más apta. Se inocularon pollos de 15 días de edad con 10^4 dosis infectantes 50% de una vacuna comercial de bronquitis infecciosa, serotipo Arkansas a través de las vías ocular y nasal y se caracterizaron las secuencias del gen S1 de los virus de bronquitis infecciosa que se encontraban en el líquido lagrimal, en la tráquea y en el tracto reproductivo de pollos individuales en diferentes tiempos de inoculación. El fenotipo predominante del virus de la bronquitis infecciosa contenido en la vacuna (antes de la inoculación) se convirtió en una población menor o no detectable en todos los tiempos de observación y en todos los tejidos después de la replicación en la mayoría de los pollos, lo que corrobora nuestras observaciones previas. Se detectaron en los tejidos o en fluidos de los pollos vacunados individualmente, cinco nuevas poblaciones predominantes designadas como componente (C) 1 al C5, que mostraban diferentes cambios no sinónimos, es decir, cambios de nucleótidos que resultan en la codificación de aminoácidos diferentes y por lo tanto en un cambio fenotípico de la población del virus predominante. Debido a las diferentes propiedades bioquímicas de algunos aminoácidos que cambiaron en la glicoproteína S1, se anticipa que ese cambio fenotípico se produjo durante el proceso de invasión. Se detectaron diferencias significativas en la incidencia de algunas poblaciones predominantes del virus de la bronquitis infecciosa en tejidos y en líquidos, por ejemplo, el fenotipo C1 mostró la mayor incidencia en el aparato reproductor de las gallinas, alcanzando una diferencia significativa frente a su incidencia en la tráquea ($P < 0.05$). Estos resultados indican por primera vez que el virus de la bronquitis infecciosa sufre variaciones intraespaciales durante la invasión del huésped, es decir, el genotipo/fenotipo dominante sufre cambios durante la invasión en el huésped de acuerdo a la forma en como el microambiente de los diferentes tejidos ejerce una presión selectiva sobre la población del virus que se está replicando.

Key words: infectious bronchitis virus, coronavirus evolution, chicken, genetic variation, phenotype variation

Abbreviations: Ark = Arkansas; C = component; DPI = days postinoculation; EID_{50} = 50% embryo infectious dose; IBV = infectious bronchitis virus; RT = reverse transcription; S = spike protein; S1 = S1 subunit; SARS = severe acute respiratory syndrome

Avian infectious bronchitis virus (IBV) belongs to the group 3 of the *Coronavirus* genus along with other avian coronaviruses (9). Coronavirus genetic variation is generated by nucleotide insertions, deletions, or point mutations made by the viral polymerase lacking proofreading capabilities and/or recombination events occurring during virus replication (20,21,22). In evolution, the mechanisms responsible for genetic variability constitute Mayr's ''step one'' (26), ''the production of variation in every generation, that is, suitable genetic or phenotypic variants that can serve as the material of selection and this will then be exposed to the process of selection.'' ''This first step of variation is completely independent of the actual selection process'' (26). In Mayr's second step, ''the genetic endowment of the few survivors during reproduction, and thus abundant new genotypes, are then tested in the next generation. Any individual favored by that selection would contribute genotypes to

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Table 1. Primers used for amplification by RT-PCR and sequencing of a portion of the S1 gene.

Name	Sequence	Orientation	Position[A]	Ref.
NEWS1OLIGO5′	5′-TGA AAC TGA ACA AAA GAC-3′	Forward	−66 to −48	16
S1OLIGO3′	5′-CAT AAC TAA CAT AAG GGC AA-3′	Reverse	1675–1656	23
S1R	5′-CAT CTG AAA AAT TGC CAG-3′	Reverse	742–725	39
S17F	5′-TGA AAA CTG AAC AAA AGA CCG ACT TAG-3′	Forward	−66 to −40	
S18R	5′-GGA TAG AAG CCA TCT GAA AAA TTG C-3′	Reverse	752–728	

[A]Position in Ark vaccine S-coding sequence.

the gene pool that were apt to spread in future generations and thus enhance the adaptation of the population as a whole".

The S1 subunit (S1) of the spike protein (S) of IBV is responsible for viral attachment to cells and is a primary target for host immune responses as it induces virus neutralizing- and hemagglutination inhibition antibodies (6,7,8,10,17,18,29). The role of S1 in determining the species and tissue/cell tropism of several coronaviruses, including IBV, has been reported extensively (2,3,5,12, 13,14,24,25,31,34,40). Because of the relevance of S1 for IBV's successful replication and immunologic escape, the extensive variation exhibited by the S1 glycoprotein among IBV populations (21,22) is probably the most relevant phenotypic characteristic for this virus's "adaptation" and evolutionary success in the environment.

We have reported previously the different degrees of genetic heterogeneity among four commercial ArkDPI-derived IBV vaccines before passage in chickens, reflected in the nucleotide sequences encoding the S1 subunit of the S protein. Within 3 days after inoculation into chickens, subpopulations with an S gene sequence distinct from the vaccine predominant consensus were found in several organs, including tears, trachea, and Harderian glands (39). These findings suggested that a distinct virus subpopulation was positively selected by the chicken upper respiratory tract. Results supporting that Ark-type IBV vaccine strains undergo selection in chickens after vaccination also have been reported by others (27).

In the chicken host, IBV initially replicates in the upper respiratory tract producing lesions in epithelial cells (32) of the nostrils, Harderian gland, trachea, lungs, and air sacs (28,35,37). After a short viremia the virus invades distant tissues including the kidneys, the urogenital tract, and the gastrointestinal tract (e.g., 36). We hypothesized that during host invasion, the replicating IBV population is confronted with distinct selective pressures in the different tissues, leading to selection of the most fit subpopulation. To confirm this hypothesis, we inoculated chickens with an Ark-type IBV via the ocular and nasal routes and analyzed the S1 gene sequences of IBV contained in different tissues and at different times postinoculation.

MATERIALS AND METHODS

IBV. A commercially available single-entity live attenuated Ark-type IBV vaccine strain was used. The lyophilized vaccine was reconstituted in sterile tryptose broth and used for inoculation of chickens.

Chickens. Seventy-two white leghorn chickens, hatched from specific pathogen-free fertile eggs (Sunrise Farms, Catskill, NY) were used to evaluate IBV intraspatial variation. Hatched chickens were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

Experimental design. All chickens were inoculated with 100 μl containing a 10^4 50% embryo infective dose (EID_{50}) of IBV via the ocular (25 μl in each eye) and nasal (25 μl in each nostril) routes at 15 days of age. Twenty-four chickens were euthanized at 6, 9, and 20 days postinoculation (DPI), and samples of trachea, oviduct or testicles, kidney, and cecal tonsil were collected from each chicken. Samples of tear fluid were obtained from each chicken before euthanasia as described (38).

RNA extraction. Viral RNA was prepared from tear samples of each chicken using a QIAmp viral RNA mini kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Total RNA was isolated from tracheal, kidney, cecal tonsil, oviduct, or testicle homogenates from each chicken using the Tri-Reagent RNA isolation reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's protocol.

Reverse transcription (RT)-PCR. cDNA for sequencing a portion of the IBV S1 gene was prepared from RNA by RT-PCR using a One Step RT-PCR kit (QIAGEN) and primers NEWS1OLIGO5′ and S1OLIGO3′, NEWS1OLIGO5′ and S1R, or S17F and S18R Table 1; (16,23,39). For 24 of the samples, two or three separate RT-PCRs were carried out, and the products were sequenced separately. The products obtained were visualized by SYBR Green staining after agarose gel electrophoresis. If inadequate amounts of cDNA for sequencing were obtained, the cDNA was further amplified by PCR using a Platinum PCR Supermix kit (Invitrogen, Carlsbad, CA) and primers S17F and S1R (for NEWS1OLIGO5′/S1R RT-PCR products) or NEWS1-OLIGO5′ and S1R primers (for NEWS1OLIGO5′/S1OLIGO3′ RT-PCR products). In total, 12 sequences were obtained by this method.

Sequencing of cDNA generated by RT-PCR or RT-PCR plus PCR. After verification by agarose gel electrophoresis, amplified cDNA was purified using the QIAquick PCR purification kit (QIAGEN) and submitted to the Massachusetts General Hospital DNA core facility for sequencing using primers S1R, S17F, and/or S18R. Thus, the sequence of the first approximately 720 nucleotides of the S1 coding sequences was obtained. Sequences were assembled and aligned using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). Sequences not already present in GenBank were submitted under the accession numbers GQ484957 to GQ484963. Sequence chromatograms were examined to identify nucleotide positions with more than one nucleotide, indicating the presence of relevant levels of more than one subpopulation. For sequences showing detectable levels of more than one subpopulation, the populations were assigned to their predominant population based on the major nucleotide peaks.

Incidences of subpopulations were calculated, and the chi-square test was used to determine statistical differences between and within groups. Differences with P values <0.05 were considered significant.

RESULTS

IBV S1 gene amplification. As shown in Table 2, a portion of the S1 gene of IBV was consistently amplified from tear fluid (44/48 samples) and tracheal samples (32/48) from inoculated chickens 6 and 9 DPI but rarely at 20 DPI. In contrast, viral S1 sequences were successfully amplified from fewer oviduct or testis samples (9/72) and rarely from kidneys (2/72) and cecal tonsils (2/72).

Table 2. S1 gene sequences obtained from tissues or tear fluids of 24 chickens on days 6, 9, and 20 after inoculation.

	No. of sequences		
	Day 6	Day 9	Day 20
Tear fluid	23	21	4
Trachea	18	14	3
Oviduct/testis	8	0	1
Cecal tonsil	2	0	0
Kidney	1	0	1

IBV S1 gene sequences in inoculated chickens. IBV-Ark found in tissues or fluids of individual inoculated chickens exhibited differences in S1 gene consensus sequences. Nucleotide and amino acid positions in which differences were observed in more than one chicken are shown in Table 3. Only those differences representing nonsynonymous changes, i.e., resulting in different amino acids encoded and therefore in a phenotypic change of the predominant virus population, are shown in Table 3. (Synonymous changes were not consistently found.) Six distinct virus predominant populations are shown, including the vaccine parent before inoculation. The five positively selected distinct predominant populations were designated component (C) 1 through 5, i.e., C1, C2, C3, C4, and C5. Compared with the vaccine parent, C3 exhibited nucleotide and amino acid changes in six positions, the most among the five newly selected populations. Therefore, C3 showed the largest phenotypic distance to the vaccine's predominant population. In addition to the number of amino acid changes, the character of the four unique changes adds phenotypic distance to the predominant population of the IBV vaccine strain before inoculation. The hydrophobic amino acid leucine at position 76 is replaced by the bulkier hydrophobic amino acid phenylalanine in C3. At amino acid position 119, serine is replaced by proline in C3, an amino acid that has a significant effect upon the orientation of the polypeptide chain. At positions 171 and 198, amino acid substitutions in C3 involve a change from an uncharged amino acid side chain to a positively charged side chain and vice versa. C1 and C4 each exhibited three nucleotide and amino acid changes, C2 showed two changes, and C5 showed one change in this portion of the S1 sequence. The change at position 43 from hydrophobic tyrosine to positively charged histidine in virus populations C1, C2, C3, and C4 could potentially result in a different folding of the polypeptide chain and/or interaction with receptor.

Most of the samples (89%) showed a single or vastly predominant S1 nucleotide sequence. The rest (10 samples) showed more than one nucleotide peak at specific positions, indicating mixed populations. Such populations were assigned to their predominant population based on the major nucleotide peaks for the analyses to follow. Seven of these sequences were very nearly 50% each of two sequences and were assigned so that they would proportionally add to the incidence of each subpopulation. For 22 of the 24 samples (92%) that were amplified and sequenced more than once, the same subpopulation(s) was detected each time, verifying that the amplified cDNA reliably represents the IBV population in each sample.

In addition to the six populations shown in Table 3, five additional variants (GenBank accessions GQ484959–GQ484963) were each identified as the single IBV population in one or more samples from a single chicken. Two variants each differed from the vaccine parent population in one amino acid position, one variant differed from population C1 in one position, one from population C3 in one position, and one from population C3 in two amino acid positions. For the analyses to follow, each of these variant populations was grouped with the population to which it was most similar.

Incidence of IBV subpopulations in chickens (all tissues) by day after inoculation. The incidence of the distinct IBV subpopulations, including the vaccine parent, in the chicken samples is shown in Fig. 1. As seen in this figure, the vaccine parent predominant population was not detected at day 6 DPI in any of the sampled chickens ($n = 24$), demonstrating rapid negative selection of this phenotype. In contrast all newly selected phenotypes, C1 through C5, were detected at varying frequencies in the chickens. At 6 DPI, C4 was the most frequent predominant population in the birds (45.3% of the samples) followed by C1 with 26.7%. A similar pattern was observed at 9 DPI, with 52.5% of the positive samples showing C4 as the predominant population followed by C1 with 15%. The vaccine parent was detected in 11.3% of the positive samples at 9 DPI. Other populations (C2, C3, and C5) showed incidences varying between 6.3% and 7.5%.

Populations by tissue or fluid (all days). Only IBV populations detected in tear fluid, trachea, and the reproductive tract were further evaluated due to the small number of S1 sequences obtained

Table 3. Differences in amino acids encoded by S1 gene of Ark-type IBV parent strain and predominant populations detected in tissues of vaccinated chickens.

	Nucleotide position	127	167	226	355	388	511	593	637	
Designation	Amino acid position	43	56	76	119	130	171	198	213	GenBank accession[A]
Vaccine parent	Nucleotide	T	A	C	T	A	T	A	T	EU359644
	Amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Ser	
C1[B]	Nucleotide	C	A	C	T	G	T	A	G	EU359643
	Amino acid	His	Asn	Leu	Ser	Gly	Tyr	Lys	Ala	
C2	Nucleotide	C	A	C	T	A	T	A	G	EU359650
	Amino acid	His	Asn	Leu	Ser	Ser	Tyr	Lys	Ala	
C3	Nucleotide	C	A	T	C	A	C	C	G	EU359626
	Amino acid	His	Asn	Phe	Pro	Ser	His	Thr	Ala	
C4	Nucleotide	C	G	C	T	A	T	A	G	GQ484957
	Amino acid	His	Ser	Leu	Ser	Ser	Tyr	Lys	Ala	
C5	Nucleotide	T	A	C	T	A	T	A	G	GQ484958
	Amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Ala	

[A]The complete S1 or S sequences in GenBank were obtained from studies independent from the present study. The sequences in the present study match the GenBenk sequences in nucleotide positions 1–724. Sequences beyond nucleotide 724 were not determined in the present study.

[B]Selected populations were designated component (C) 1 through C5.

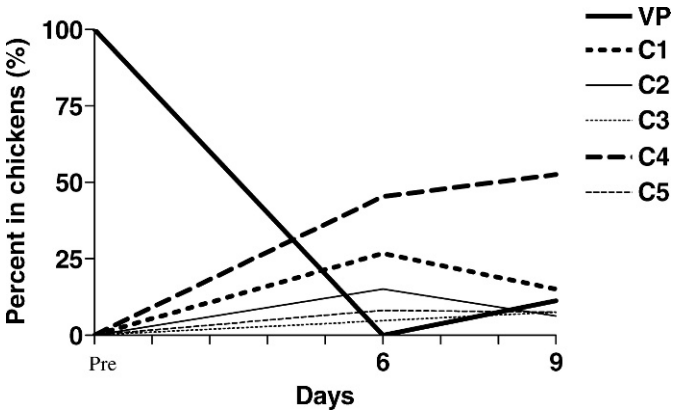

Fig. 1. Incidence of IBV subpopulations in chickens (all tissues) by day after inoculation. The vaccine predominant phenotype (VP) before inoculation (Pre) is rapidly negatively selected after replication in the host. Other subpopulations designated component (C) 1 through C5 became predominant in chicken tissues and tear fluid. C4 and C1 were the most frequent subpopulations found on days 6 and 9 after inoculation.

in kidneys and cecal tonsils. As seen in Fig 2A, C1 and C4 were the most frequently found predominant phenotypes in the tear fluid of inoculated birds. Their incidence was significantly higher ($P < 0.05$) than the incidence of any of the other phenotypes. No significant differences were detected among the incidence of all other populations ($P > 0.05$). In the trachea, the most frequent predominant population was C4 (50%), whose incidence was significantly higher ($P < 0.05$) than all other populations (Fig. 2B). The vaccine parent, C1, C2, and C5 showed incidences varying between 22% for C2 and 4.9% for the vaccine parent. C3 was not detected in the trachea of any chickens. The results of the reproductive tract (Fig. 2C) include the S1 gene sequences ($n = 9$) detected in the oviduct of female and testis of male birds. These results were grouped because both represent a similar distance from the respiratory tract even though we are aware that the microenvironments, and thus the exerted selective pressure, may differ between these distinct tissues. As seen in Fig. 2C, the most frequent phenotype detected in the reproductive tract was C1 (44%) followed by C4 and C5 with 22% incidence each. Neither the vaccine parent nor C2 was detected in these tissues.

Incidence of IBV subpopulations among tissues or fluids within the host. The incidences of the predominant phenotypes in samples of tissues or fluids within the chicken are compared in Fig. 3. The fact that some comparisons did not achieve statistical significance by chi-square test is probably due to the number of amplicons sequenced. As seen in this figure, phenotype C1 showed the highest incidence in the reproductive tract of the chickens, achieving a significant difference versus its incidence in the trachea. C1 also showed a clear tendency to be more frequently found in the tear fluid than in the trachea ($P = 0.07$). A significant difference also was detected for C3, which was found in approximately 10% of samples in tear fluid and in the reproductive tract but was absent in the trachea. C4 was detected with similar frequency in the tears and trachea with a clear tendency for lower frequency in the reproductive tract. However, this latter difference did not achieve statistical significance. C5's incidence was highest in the reproductive tract, with a significant difference versus its incidence in the tears ($P < 0.05$). It was interesting to notice a tendency for a decrease the incidence of the vaccine parent phenotype in the order tears (8.3%), trachea (4.9%), and reproductive tract (0%). An opposite pattern was observed with phenotype C5, which tended to increase toward the reproductive tract (22%) versus 4.2% in the tear fluids and 11% in the trachea.

Distinct IBV subpopulations in different tissues of individual chickens were found in a high proportion of animals. For example, in 10/20 (50%) chickens at 6 DPI and in 6/12 (50%) chickens at 9 DPI, a different predominant IBV population was found in tear versus tracheal samples.

Time-dependent kinetics of IBV subpopulations. The incidences of each IBV predominant subpopulation in the tears, trachea, and reproductive tract did not vary significantly between days 6 and 9 after inoculation. A comparison of the incidence of each IBV subpopulation in the trachea is shown in Fig. 4 as an example.

DISCUSSION

We have demonstrated previously that there are genetic differences among Ark-type-attenuated IBV vaccine strains. We also know both the genetic (S1 gene sequence) and phenotypic (deduced amino acids) characteristics of the S1 glycoprotein of the predominant viral subpopulation selected once this virus strain has replicated in the environment of the upper respiratory tract (39). For

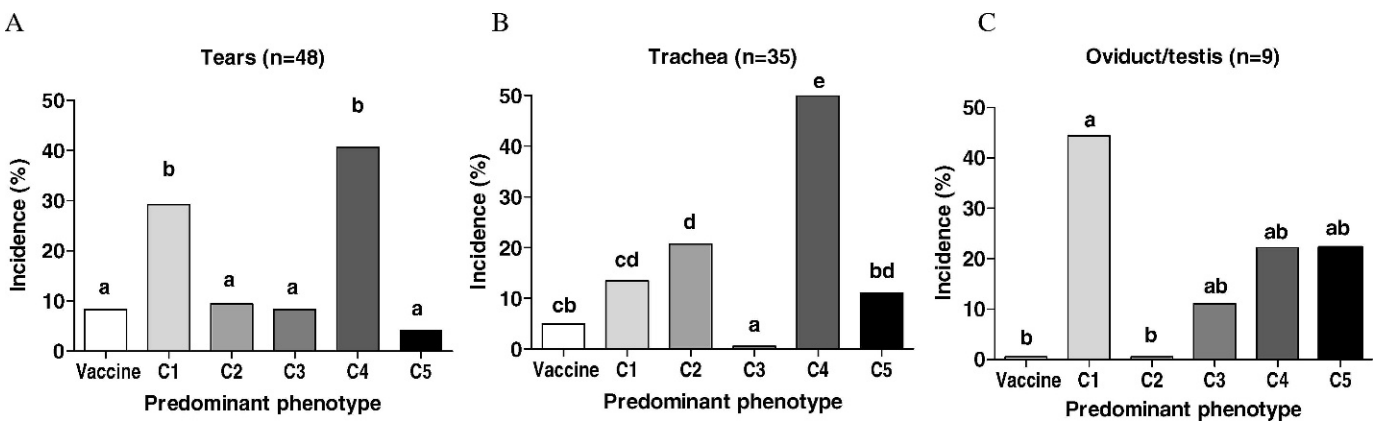

Fig. 2. Incidence of IBV subpopulations (bars) in tear fluid (A), trachea (B), or reproductive tract (C) in chickens after inoculation (considering all days tested) with an Ark-type vaccine strain. The number of samples containing distinct S1 gene sequences was compared by chi-square test. Significant differences ($P < 0.05$) are indicated by different letters above bars.

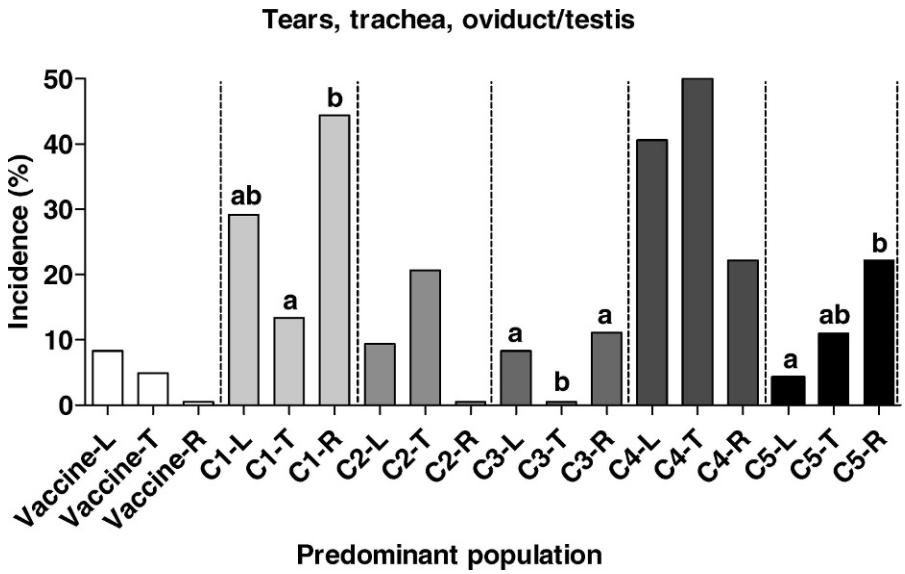

Fig. 3. Incidences of IBV subpopulations (bars) in tear fluid (-L), trachea (-T), or reproductive tract (-R) within chickens after inoculation (considering all days tested) with an Ark-type vaccine strain. The number of samples containing distinct S1 gene sequences was compared by chi-square test. Significant differences ($P < 0.05$) are indicated by different letters above bars.

these reasons, and because others also have shown rapid selection of Ark-type IBV strains in chickens (27), we chose an Ark-type vaccine strain for the current experiments.

Viral RNA was consistently amplified from samples collected at 6 and 9 DPI but rarely at 20 DPI. This result can be explained by the low virulence of the IBV strain used in these experiments, i.e., virulent phenotypes to the natural host have been negatively selected during multiple generations via the process of ''attenuation in chicken embryos'' or adaptation to the chicken embryo host. However, such an adapted virus population still shows genetic heterogeneity (39), and there is no reason to assume that the mechanisms to generate diversity could have been compromised during the artificial adaptation process.

When analyzing the incidence of the vaccine parent predominant phenotype (Fig. 1) in the chickens 6 days after inoculation, it becomes clear that it was negatively selected. This result corroborates our previous findings for other ArkDPI-derived vaccines as well as findings by others (27,39).

Nucleotide alterations of the S1 coding sequences of coronaviruses have been shown to be associated with organ tropism and *in vivo* pathogenesis (19,33). During adaptation of IBV to Vero cells or attenuating passages in embryonated eggs, more changes accumulate in the S gene than in other genes (1,12). In the present study, five new predominant IBV phenotypes were detected in the host tissues or fluids. The new predominant populations showed varying degrees of phenotypic distance to the predominant population of the vaccine parent. Differences varied from populations exhibiting as many as six nonsynonymous changes to populations showing only one change within the region sequenced (Table 3). However, sequences of the entire S1 coding sequence of populations selected from this vaccine in an unrelated experiment indicate additional changes in the portion of the sequence not determined in the present study. The subpopulation showing only one nonsynonymous change with the vaccine parent in the region of the S1-coding sequences determined in the present study has two additional non-synonymous differences for a total of three amino acid differences in S1 (V. van Santen,

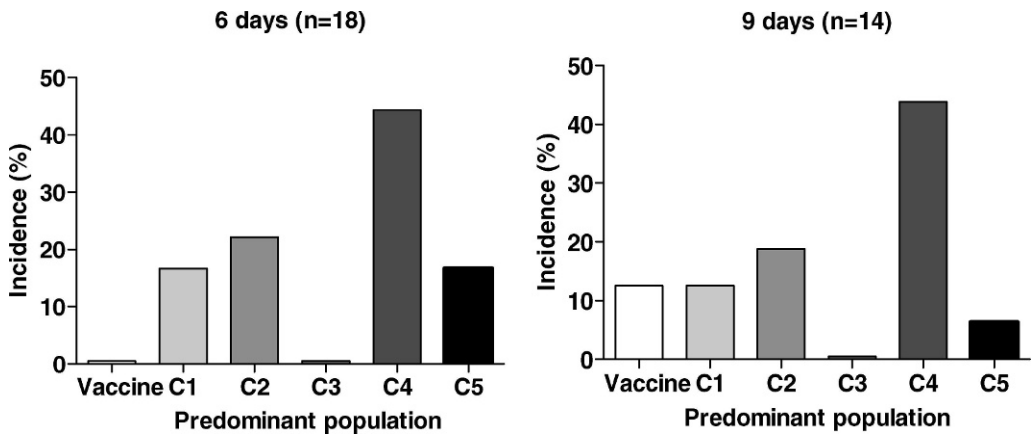

Fig. 4. Incidence of IBV subpopulations (bars) in the tracheas chickens at 6 and 9 days after inoculation with an Ark-type vaccine strain. Incidences of the predominant phenotypes in the tracheas on the 2 days were compared by chi-square test. No significant differences were detected between days 6 and 9.

unpubl. data). In addition, some of these changes were qualitatively essential as they probably changed local charge and/or morphologic properties of the S1 glycoprotein (Table 3). In the severe acute respiratory syndrome (SARS) coronavirus, a single amino acid change in the S protein of a palm civet isolate markedly increased its affinity for the human SARS coronavirus receptor (25). However, the impact of such amino acid changes in IBV on attachment to host cells or immunologic escape requires further investigation.

During the course of a previous study, we used primers designed to specifically amplify a portion of the S1 coding sequences of ArkDPI subpopulations selected in chickens to demonstrate the presence of potentially selectable subpopulations in the vaccine used in the present study (39). The specific primers would recognize C1–C4 but would recognize neither the major vaccine population nor C5. Sequence chromatograms of the RT-PCR products were consistent with the presence of C1–C4 in the vaccine before replication in chickens. A quantitative analysis of the nucleotide peak heights in the chromatograms at positions where populations C1, C3, and C4 exhibit unique nucleotides indicated that C3 was present in the highest proportion (approximately 40%), with C4 (approximately 30%) and C1 (approximately 20%) following. C2 cannot be directly estimated because it has no unique nucleotides, but it might comprise the remaining approximately 10% of potentially selectable virus in the vaccine. The relative frequencies of selection of subpopulations C4, C1, and C2 in tears of the current study correspond to their previously determined relative frequencies in the vaccine. However, C3 is selected at a lower frequency in all tissues than expected based on its relative frequency in the vaccine compared to C1, C2, and C4. This suggests that C3 is not as fit in the studied environments as the other selected populations.

Even though IBV targets epithelial cells, the microenvironment of the distinct tissues harboring this type of cells varies within the host. The environment of these tissues not only differs in essential physical and biochemical characteristics (e.g., the environments of the trachea and the oviduct) but also cell receptors among different tissues might vary due to, for example, alternative pre-mRNA splicing to produce distinct mRNAs that give rise to variant proteins (4,30) or due to alternative glycosylation. In addition, the immune responses that clearly exert selective pressure to invading parasites also vary between tissues (e.g., different degrees of activity of mucosal or systemic immune responses). From the perspective of the virus population, it is also conceivable that the differences observed in IBV populations involve variation in affinity of cell-receptor interactions. It is also possible that the selected changes in S1 might be linked and/or associated with changes of regulatory proteins encoded at distant sites of the viral genome that could also contribute to the fitness of the virus in distinct tissues. The current results showed that the incidence of some of the predominant phenotypes varied significantly between tissues (Fig. 3) of the inoculated chickens. It was also interesting to notice that the vaccine parent predominant phenotype showed a tendency to decrease its incidence toward the tissues located farther away from the site of initial inoculation. In contrast, other predominant populations (e.g., C5) showed the reverse tendency. These results indicate for the first time that IBV undergoes intraspatial variation during host invasion, i.e., the dominant genotype/phenotype further changes during host invasion as the microenvironment of distinct tissues exert selective pressure on the replicating virus population.

In the present study no significant differences were detected in the incidence of predominant phenotypes within the same tissue between 6 and 9 days after inoculation. We speculate that the time span evaluated (3 days) was not long enough to allow selection. However, it is conceivable that further maturation of the specific immune responses, resulting, for example, in improved affinity of immune molecules, may have an impact on selection of virus subpopulations.

From an applied perspective, these results strongly suggest that viruses to be used for phylogenetic studies should be obtained from the same tissues of the chickens. Otherwise, intraspatial variation may be adding variation to such data.

Evolution of viruses by selection of the fittest subpopulations can lead to the selection of virulent viruses and the emergence of new viral pathogens (11). It seems reasonable to assume that intraspatial variation is also contributing to this complex phenomenon and to related problems, such as reversion to virulence of attenuated IBV vaccine strains after chicken passages (15).

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