

S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000)

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The S1 genes of isolates of avian coronavirus infectious bronchitis virus (IBV) from commercial chickens in the US and Israel (20 isolates from each country) were studied using reverse transcription-polymerase chain reaction restriction fragment length polymorphism and sequencing. Partial sequences spanning the amino terminus region of S1 from amino acid residues 48 to 219, based on the Beaudette strain, were used for analysis. Phylogenetic clustering and high-sequence identity values were used to identify isolates that appeared to be derived from live IBV vaccines used in the two countries. Novel variant strains, unrelated by S1 sequencing and restriction fragment length polymorphism analyses to reference and vaccine strains, were also identified. Based on S1 sequence identity to available vaccines, the potential to use vaccination to control IBV infections was evaluated. Vaccination with commercial live strains Massachusetts (Mass), Arkansas (Ark) or DE/072/92, generally produced immunity against vaccine-related field isolates displaying high S1 sequence similarities ($\geq 90\%$) to the respective vaccine strains. Immunization with a bivalent vaccine containing the Mass and Ark strains provided good cross-protection, averaging 81% against challenge with five variant isolates from the US having amino acid identity values ranging from 62 to 69% to Mass and from 68 to 83% to Ark, respectively. In contrast, the H120 vaccine strain induced low levels of protection, ranging from 25 to 58% against variant field isolates from Israel with amino acid identity values from 65 to 67%.

Introduction

Infectious bronchitis (IB) is among the most common and difficult of all poultry diseases to control. IB is highly contagious and results in significant economic losses in commercial broiler, layer and breeder chickens. The causative coronavirus, infectious bronchitis virus (IBV), frequently causes respiratory disease in young chickens and egg production losses in hens. In addition, some strains of the virus exhibit a renal (kidney) tropism and produce up to 30% mortality in affected flocks.

IBV readily undergoes mutation in chickens resulting in the emergence of new variant serotypes and genotypes. Mutations occur frequently in hypervariable regions of the S1 subunit of the envelope spike (S) glycoprotein gene. The S1 protein is responsible for infection of the host cell (Cavanagh & Davis, 1986; Koch *et al.*, 1990; Ignjatovic & Galli, 1994), inducing virus-neutralizing antibody (Cavanagh *et al.*, 1988) and immunity (Cavanagh *et al.*, 1997). New variant strains may differ as much as 55% in their S1 amino acid sequence compared with vaccine strains (Kusters *et al.*, 1989; Gelb *et al.*, 1997). Many “new” variants have been isolated from commercial flocks (Davelaar *et al.*, 1984; Kusters *et al.*, 1989; Gelb *et al.*, 1991, 1997, 2001; Cavanagh *et al.*, 1992; Gough *et al.*, 1992, 1996; Capua *et al.*, 1994; Moore *et al.*, 1998; Fabio *et al.*, 2000; Lee

& Jackwood, 2001; Zeigler *et al.*, 2002; Schikora *et al.*, 2003; Meir *et al.*, 2004). Two genetic-based strategies have been used in the US and Israel to identify field strains; restriction fragment length polymorphism (RFLP) analysis (Kwon *et al.*, 1993; Meir *et al.*, 2004), and reverse transcription-polymerase chain reaction (RT-PCR) product cycle sequencing (Kingham *et al.*, 2000).

The purpose of this research was to characterize 40 IBV isolates from diseased flocks in the US and Israel by partial sequencing of the S1 protein. Vaccine strains with the highest sequence identity to the field isolates were evaluated for their ability to protect chickens in challenge of immunity trials.

Materials and Methods

Viruses. Twenty IBV field isolates from the US (Table 1) and an equal number from Israel (Table 2) were recovered from commercial meat-type (broiler) and egg-type (layer) chicken flocks between 1996 and 2000. The isolates as well as reference strains for Arkansas (Ark) DPI, Connecticut (Conn), DE/072/92 (Delaware 072), JMK, and Massachusetts (Mass) 41 are maintained as allantoic fluid seed stocks in the authors' laboratories.

Commercially available live IBV vaccines currently licensed for use in the US and Israel were used to evaluate their efficacy against challenge with selected field isolates recovered from the respective countries.

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Received 17 November 2004. Accepted February 2005

ISSN 0307-9457 (print)/ISSN 1465-3338 (online) © 2005 Houghton Trust Ltd
DOI: 10.1080/03079450500096539

Table 1. *IBV field isolates from the US*

IBV field isolate identity	Year of isolation	Location	Type of chicken	Form of disease	Genbank accession number
PA/4327/97	1997	Pennsylvania	Broiler	Respiratory and renal	AY789943 AY789944
PA/Wolgemuth/98	1998	Pennsylvania	Broiler	Renal	AF305595
PA/1220/98	1998	Pennsylvania	Layer	Respiratory	AF200685
PA/5173/98	1998	Pennsylvania	Layer	Respiratory and renal	AY789946
PA/5344/98	1998	Pennsylvania	Layer	Respiratory	AY789947
CA/510/99	1999	California	Broiler	Respiratory	AY789932
CA/1249/99	1999	California	Layer	Respiratory	AY789931
CA/3402/99	1999	California	Layer	Renal	AY789933
DE/51/99	1999	Delaware	Broiler	Respiratory	AY789936
DE/322/99	1999	Delaware	Broiler	Respiratory	AY789934
DE/406/99	1999	Delaware	Broiler	Respiratory	AY789935
GA/5425/99	1999	Georgia	Layer	Reproductive	AY789937
MD/183/99	1999	Maryland	Broiler	Respiratory	AY789939
MD/30/99	1999	Maryland	Broiler	Respiratory	AY789940
MN/11375/99	1999	Minnesota	Layer	Reproductive	AY789941
PA/171/99	1999	Pennsylvania	Layer	Renal	AF419314
PA/5083/99	1999	Pennsylvania	Layers	Reproductive and renal	AY789945
AL/2975/00	2000	Alabama	Broiler	Respiratory	AY789929
AL/3701/00	2000	Alabama	Broiler	Respiratory	AY789930
MD/106/00	2000	Maryland	Broiler	Respiratory	AY789938

Vaccines containing serotypes from the US were Mass, Ark, and DE/072/92. A commercial H120 strain vaccine from Israel was used. The S1 genes of the vaccines and reference strains were characterized by RFLP and sequencing to provide a basis of comparison with the field isolates.

Field isolates and vaccines used for S1 RFLP and sequencing were inactivated by treating approximately 2 ml allantoic fluids or rehydrated lyophilized vaccines with an equal volume of molecular biology-grade phenol (pH 4.3) (Fisher Scientific, Fairlawn, New Jersey, USA). Following inactivation, field isolates and vaccines from both countries were exchanged for S1 gene analyses. Sequencing was performed in the US and RFLP analysis was performed in Israel.

S1 gene RFLP analysis. IBV RNA was extracted using a kit (QIAamp Viral RNA Mini Kit; Qiagen, Inc., Valencia, California, USA). RT-PCR RFLP was performed to amplify the complete S1 gene using forward primer S1 OLIGO5' and reverse primer S1 OLIGO3', yielding an amplicon of approximately 1700 nucleotides (Kwon *et al.*, 1993; Meir *et al.*, 2004). PCR products were digested with three commonly

used restriction enzymes: *Bst*YI, *Hae*III, and *Xcm*I (Kwon *et al.*, 1993). Digested PCR products were separated by electrophoresis in 2% agarose gels stained with ethidium bromide. Restriction patterns of the field isolates were compared with patterns observed with digested vaccine and reference strain PCR products.

S1 gene PCR product cycle sequencing. Viral RNA was extracted (QIAamp Viral RNA Mini Kit; Qiagen, Inc.) from allantoic fluid stocks of the field isolates or directly from the vaccines. RNA was eluted in sterile diethyl pyrocarbonate-treated water and stored at -70°C until used for RT-PCR.

RT was performed on viral RNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, California, USA). Approximately 2 μl extracted viral RNA was used to synthesize cDNA. Amplification of the 5' end of the S1 gene was performed using the forward primer S1 OLIGO5' (Kwon *et al.*, 1993) and the reverse primer CK2 (Keeler *et al.*, 1998), resulting in an amplicon of approximately 700 nucleotides. PCR was performed as described previously (Kingham

Table 2. *IBV field isolates from Israel*

IBV field isolate identity	Year of isolation	Type of chicken	Form of disease	Genbank accession number
IS/188/96	1996	Broiler	Renal	AY789949
IS/222/96	1996	Broiler	Respiratory	AY793382
IS/223/96	1996	Broiler	Respiratory	AY789950
IS/236/96	1996	Broiler	Respiratory	AY789951
IS/251/96	1996	Broiler	Respiratory	AY789952
IS/287/96	1996	Broiler	Respiratory	AY789953
IS/64714/96	1996	Broiler	Respiratory	AY789965
IS/361/97	1997	Broiler	Renal	AY789954
IS/373/97	1997	Broiler	Respiratory	AY789955
IS/378/97	1997	Broiler	Renal	AY789956
IS/385/97	1997	Broiler	Renal	AY789957
IS/388/97	1997	Broiler	Respiratory and renal	AY789958
IS/415/97	1997	Layer	Respiratory	AY789959
IS/572/98	1998	Broiler	Respiratory	AY789961
IS/585/98	1998	Broiler	Renal	AY789962
IS/589/98	1998	Broiler	Respiratory	AY789963
IS/617/98	1998	Broiler	Respiratory	AY789964
IS/665/98	1998	Broiler	Respiratory	AY789966
IS/51355/98	1998	Broiler	Respiratory	AY789960
IS/720/99	1999	Broiler	Respiratory	AY091552

et al., 2000) with the exception that extension was performed at 60°C. PCR products were cut from 1.8% agarose gels, purified with the QIAquick Gel Extraction Kit (Qiagen, Inc.) and DNA was quantified as described (Kingham *et al.*, 2000).

Purified RT-PCR products were sequenced in the forward direction using primer S1 OLIGO5' and in the reverse direction using primer CK2 (Kingham *et al.*, 2000). Analysis was performed using partial sequences spanning the amino terminus region of S1 from amino acid residue 48 to 219, based on the Beaudette strain (GenBank accession number NP 04083). The gene fragment encompasses hypervariable region 1 and the majority of hypervariable region 2 (Cavanagh *et al.*, 1988; Kusters *et al.*, 1989). Analysis of the partial S1 sequences obtained by our laboratory and from GenBank and EMBL databases was performed using the CLUSTAL V (Higgins & Sharp, 1988) package of DNASTar (MegAlign, Version 1.03, 1993; DNASTar, Inc., Madison, Wisconsin, USA).

Comparison of partial and complete S1 sequences of highly related strains will yield virtually identical S1 amino acid identity values. In the case of variant strains that differ extensively in their S1 amino acid sequences, the identity values for the partial sequences will be on average, approximately up to 9% lower than for the complete S1 sequences. The lower identity of isolates using analyses of the partial sequences is a reflection of the higher relative variability associated with the 5' portion of the gene compared with that of the full-length S1 gene.

Vaccination-challenge of immunity trials. Three vaccination-challenge of immunity trials were performed. Trial 1 evaluated the efficacy of commercial live IBV vaccines containing the Ark DPI, DE/072/92, or Mass strains versus challenge with US field isolates displaying a high degree of S1 identity ($\geq 91\%$) to the vaccines. Trial 2 examined the efficacy of a commercial vaccine containing the Mass and Ark strains versus challenge with variant field isolates CA/510/99, PA/171/99, PA/5083/99, PA/5344/98, and MN/113575/99 from the US. Trial 3 assessed the efficacy of a commercial H120 strain vaccine versus challenge against field isolates IS/64714/96, IS/385/97, IS/585/98, IS/665/98, and IS/720/99 from Israel.

The procedures for each of the three vaccination-challenge trials were as follows. Two-week-old specific pathogen free (SPF) Leghorn chickens were obtained (Charles River SPAFAS, North Franklin, Connecticut, USA) and assigned to groups of 10 to 13 birds per group. The chickens were vaccinated via eye drop with recommended doses of commercial modified live IBV vaccines and were placed in Horsfall-type isolation units. Four weeks post vaccination, additional SPF leghorns were obtained from the supplier and five birds were housed with each of the IBV challenge virus treatments as challenge controls. Vaccinated and challenge control chickens were inoculated by eye drop with approximately $10^{4.0}$ to $10^{4.5}$ EID₅₀ per chick of the field isolates. Five days after challenge, tracheal swabbing was performed on all chickens and were used for virus re-isolation attempts (Ladman *et al.*, 2002). Virus isolations were performed by a single passage in 9-day-old to 11-day-old SPF embryonated eggs via chorioallantoic sac inoculation. Percentage protection values for each treatment group were calculated to determine the level of immunity provided by vaccination against challenge with the specific field isolates.

Results

RFLP and S1 gene sequence analyses.

US Isolates. IBV field isolates from the US were placed into 15 groups based on RFLP analysis using restriction enzymes, *Bst*YI, *Hae*III, and *Xcm*I (Table 3). Six groups (A-1, A-2, A-3, B, C-1 and C-2) consisted of 11 isolates that were identified as various reference strains with identical or similar RFLP patterns. Field isolates in Group A-1 (AL/2975/00, AL/3701/00) were identical by RFLP to the DE/072/92 strain. Isolates in Groups A-2 (DE/322/99, DE/406/99, GA/5425/99) and A-3 (PA/5173/98) displayed similar but not identical RFLP patterns to the DE/072/92 genotype and were identified as DE/072/92-like. *Hae*III restriction produced an addi-

tional fragment in Group A-2 (750 nucleotides) and Group A-3 (650 nucleotides). Group B isolates PA/5083/99 and PA/4327/97 produced RFLP patterns consistent with reference strain PA/Wolgemuth/98. Group C-1 consisted of a recent field isolate PA/171/99 considered now to be a reference strain (Zeigler *et al.*, 2002). RFLP analysis of PA/171/99 and MD/106/00 (Group C-2) yielded identical *Bst*YI and *Hae*III polymorphisms but a different *Xcm*I pattern.

Isolates in six of the 15 RFLP groups could not be identified as known S1 genotypes because they were novel variants unrelated to known reference strains (Table 3). Variant IBV isolates were placed into six RFLP groups (E, F-1, F-2, H, J, and K) each containing a single isolate. All of the novel variant strains displayed unique RFLP patterns. Moreover, it was noted that isolates in Group E (CA/1249/99), Group J (MN/11375/99), and Group K (PA/1220/98) displayed S1 sequence identity values to reference strains ($\leq 75\%$) that were consistent with previous cut-offs for identifying a strain as a novel variant (Kingham *et al.*, 2000). Interestingly, novel variant isolates in Group F-1 (CA/510/99), Group F-2 (CA/3402/99), and Group H (PA/5344/98) had S1 sequence identity values to reference strains that ranged from 83 to 85%.

Finally, field isolates in Groups D, G, and I also did not display RFLP patterns consistent with vaccine or reference strain genotypes (Table 3). However, isolates in these groups had high S1 sequence identity values with vaccine or reference strain genotypes. Identity values of the isolates in Groups D (MD/183/99) and G (MD/30/99) were $\geq 98\%$ to reference strain Ark/1529/95 (Nix *et al.*, 2000). Isolate DE/51/99 of Group I had RFLP patterns similar to the JMK strain, but its sequence was consistent ($\geq 94\%$ similar) with the Mass 41 genotype.

A phylogeny of the partial S1 amino acid sequences of US field isolates, commercial vaccines and selected reference strains is shown in Figure 1. The respective reference and vaccine strains for Ark, Conn, DE/072/92, and Mass genotypes clustered together. S1 amino acid sequences of commercial vaccines and their respective reference strains were highly related, ranging from 98 to 100% identity (Table 3). The field isolates demonstrated extensive sequence variability to the reference and vaccine strains. PA/1220/98, CA/1249/99, and MN/11375/99 were highly novel variants and were distantly related to other US isolates, reference strains and vaccines. Isolates MD/30/99 and MD/183/99 were found to be highly similar ($\geq 97\%$) to Ark reference strain Ark/1529/95. PA/4327/97 and PA/5083/99 were $\geq 95\%$ similar to PA/Wolgemuth/98. PA/171/99 and MD/106/00 were 98% similar to each other. DE/51/99 was highly similar (94%) to Mass 41 and even more closely related (98 to 99%) to the Mass vaccines. Isolates AL/2975/00, AL/3701/00, DE/322/99, DE/406/99, GA/5425/99 and PA/5173/98 were related to DE/072/92, with identity values ranging from 89 to 92%.

Israeli isolates. IBV field isolates from Israel were placed into four RFLP groups (Table 4). Group A contained 10 of 20 Israeli isolates (IS/236/96, IS/287/96, IS/361/97, IS/373/97, IS/385/97, IS/388/97, IS/415/97, IS/617/98, IS/665/98, and IS/51355/98) that had RFLP patterns consistent with the Mass genotype. Group B isolates (IS/188/96, IS/222/96, IS/251/96, and IS/64714/96) were identified as Variant 1, and Group C viruses (IS/223/96,

Table 3. RFLP results of IBV field isolates from the US

RFLP group/ genotype	Isolate	RFLP fragment sizes ^a			Reference strain with highest S1 sequence identity to field isolate/GenBank accession number (identity value %)
		<i>Bst</i> YI	<i>Hae</i> III	<i>Xcm</i> I	
A-1 /DE/072/92	AL/2975/00	1720	888	1720	DE/072/92/U77298 (92)
	AL/3701/00		545 130 99		
A-2/DE/072/92 like	DE/322/99	1720	888	1720	DE/072/92/U77298 (92)
	DE/406/99		750		
	GA/5425/99		545 130 99		
A-3 /DE/072/92 like	PA/5173/98	1720	888	1720	DE/072/92 /U77298 (89)
			650		
			545		
			130		
			99		
B/PA/Wolgemuth/98	PA/Wolgemuth/98 PA/5083/99 PA/4327/97	1720	620	1035	PA/Wolgemuth/98/AF305595 (100)
			480	555	
			267		
			170		
			130 60		
C-1/ PA/171/99	PA/171/99	1720	1110	1000	PA/171/99/AF419314 (100)
			350	720	
			270		
C-2	MD/106/00	1720	1100	1600	PA/171/99/AF419314 (98)
			350	900	
			270	810	
D	MD/183/99	920	1600	1030	Ark/1529/95/AF169856 (98)
		800		670	
				420	
E	CA/1249/99	883	1225	1094	DE/072/92/U77298 (68)
		676	320	388	
			60	120	
F-1	CA/510/99	1030	820	1030	Ark DPI/AF006624 (83)
		670	470	850	
				670	
				430	
F-2	CA/3402/99	1720	670	1030	Ark DPI/AF006624 (83)
			473	680	
			386		
G	MD/30/99	780	1230	810	Ark/1529/95/AF169856 (98)
		660	284	560	
		227	200	350	
H	PA/5344/98	740	1100	630	JMK/L14070 (85)
		580	536	540	
		400		340	
I /JMK	DE/51/99	1040	970	1550	Mass 41/M21883 (94)
		680	430	170	
			320		
J	MN/11375/99	700	1420	1720	Ark DPI/ AF006624 (68)
		550	300		
		470			
K	PA/1220/98	1720	820	990	Connecticut/ L18990 (35)
			680	730	
			220		

^aRFLP fragment sizes are approximate and were calculated by visual comparison with the DNA marker and supported by gel documentation and analysis software (GeneTool; Syngene, UK).

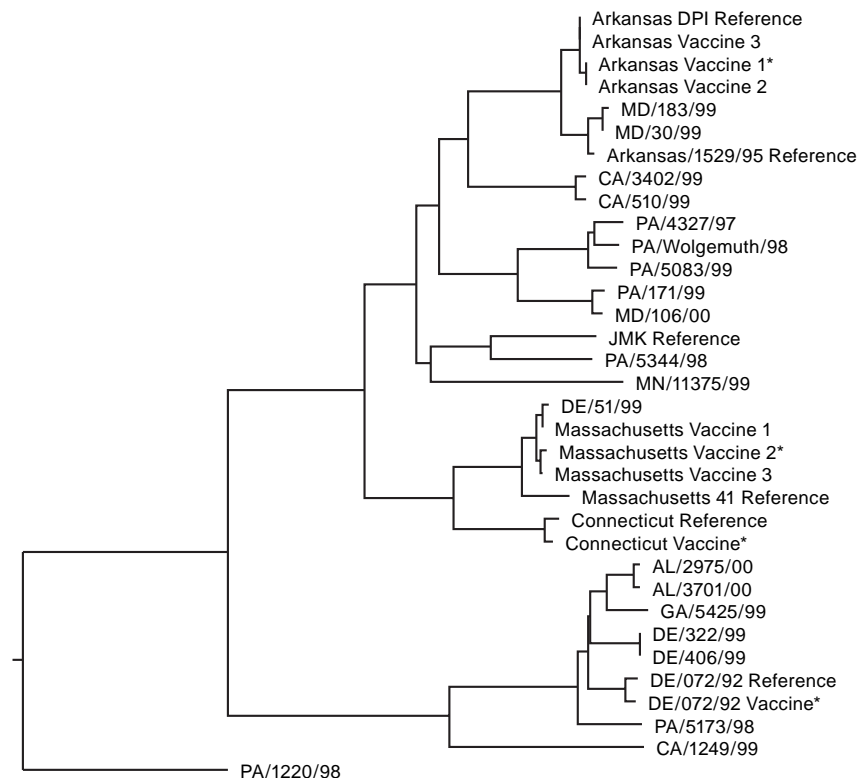


Figure 1. Phylogenetic tree showing partial (*Beaudette* GenBank accession number NP 04083 amino acid position 48 [nucleotide 144] to 219 [nucleotide 657]) S1 gene inter-relationships between IBV field isolates from the US, commercial vaccines and reference (ref) strains for Arkansas, Connecticut, DE/072/92, Massachusetts and JMK (reference strain only). * Vaccines used for immunization in the vaccination-challenge of immunity trials (Tables 5 and 6).

IS/378/97, IS/572/98, IS/585/98, and IS/589/98) were found to be Variant 2 by RFLP. One isolate, IS/720/99 (Group D) had a unique RFLP pattern and a low sequence identity to reference strains, and was therefore designated a novel variant.

S1 analysis using the partial amino acid sequences of the Israeli field isolates supported the RFLP findings (Table 4). S1 sequence identity values of the isolates identified by RFLP as Mass ranged from 95 to 100% and were highly related (95 to 100%) to the three commercial H120 vaccines. Isolates representing the Variant 1 (Group B) and Variant 2 (Group C) viruses were highly related to their respective genotypic groups, displaying S1 sequence identity values ranging from 95 to 100% and from 95 to 99%, respectively.

The S1 phylogeny of the Israeli field isolates, commercial vaccines and selected reference strains are shown in Figure 2. Nineteen of 20 isolates clustered into three clades representing Mass, Variant 1, and Variant 2 genotypes. Isolate IS/720/99, represented a new genotype.

Vaccination-challenge of immunity trials.

Trial 1: Efficacy of commercial vaccines against challenge with US field isolates with a high degree of S1 identity (>91%) to the vaccines. Vaccination with commercial IBV live strains generally conferred solid immunity against field isolates that shared a high S1 sequence identity with the field isolate used for challenge (Table 5). Vaccination with DE/072/92 resulted in partial protection (55%) against challenge with isolate DE/406/99. The isolates had S1 sequence identity values ranging from 91 to 99% to the vaccine strains used in the trial.

Trial 2: Efficacy of a commercial vaccine containing the Mass and Ark strains against challenge with variant field isolates CA/510/99, PA/171/99, PA/5083/99, PA/5344/98, and MN/11375/99 from the US. Mass and Ark strain combination vaccination produced good levels ($\geq 70\%$ protection) of immunity against challenge with variant field isolates CA/510/99, PA/171/99, PA/5083/99, and MN/11375/99 (Table 6). Vaccination with Mass and Ark produced partial protection (50%) chickens following challenge with isolate PA/5344/98. The isolates had S1 sequence identity values ranging from 62 to 69% to the Mass vaccine strain and from 68 to 83% to the Ark vaccine strain used in the trial. Only three of five (60%) of the susceptible challenge control chickens were positive for IBV following challenge with PA/5083/99, suggesting that the dose given to the controls as well as the Mass and Ark vaccinates for this treatment was low. It is possible that the level of protection (91%) in the Mass and Ark vaccinates actually may be lower than the reported value.

Trial 3: Efficacy of a commercial vaccine containing the H120 strain against challenge with field isolates from Israel (IS/64714/96 [Variant 1], IS/385/97, IS/585/98 [Variant 2], IS/665/98, and IS/720/99). Chickens vaccinated with H120 were well protected (83%) from challenge with IS/665/98, an isolate highly related to the vaccine. However, H120 protection versus challenge with IS/385/97, also highly related (96%) to H120, resulted in only partial (58%) protection. Poor protection (25 to 58%) of H120-vaccinated chickens was noted versus challenge with variant isolates IS/64714/96 (Variant 1), IS/585/98 (Variant 2) and IS/720/99. The three Israeli variant isolates used in the trial had S1 sequence

Table 4. RFLP results of IBV field isolates from Israel

RFLP group/ genotype	Isolate	RFLP fragment sizes ^a			Reference strain with highest S1 sequence identity to field isolate/GenBank accession number (identity value %)
		<i>Bst</i> YI	<i>Hae</i> III	<i>Xcm</i> I	
A/Mass	IS/236/96	1033	910	950	H120/M21970
	IS/287/96	684	470	580	(100)
	IS/361/97		325	158	(95)
	IS/373/97				(100)
	IS/385/97				(100)
	IS/388/97				(96)
	IS/415/97				(99)
	IS/617/98				(100)
	IS/665/98				(100)
	IS/51355/98				(99)
B/Variant 1	IS/188/96	1720	550	1720	Variant 1/AF093795
	IS/222/96		480		(95)
	IS/251/96		450		(100)
	IS/64714/96		240		(98)
					(100)
C/ Variant 2	IS/223/96	1230	1370	980	Variant 2/AF093796
	IS/378/97	470	350	590	(99)
	IS/572/98			150	(95)
	IS/585/98				(99)
	IS/589/98				(99)
					(99)
D/ Novel	IS/720/99	1050	1100	1000	Novel
		348	354	700	Mass 41/M21883
			332	266	(64)

^aRFLP sizes are approximate and were calculated by visual comparison with the DNA marker and supported by gel documentation and analysis software (GeneTool; Syngene, UK). When available, the full S1 gene sequence was mapped for the relevant restriction sites and compared with the recorded data.

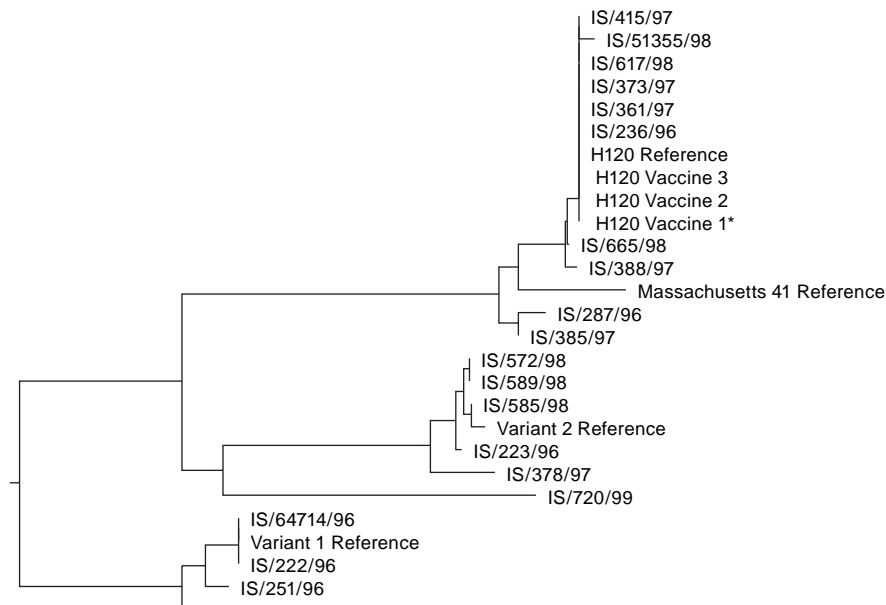


Figure 2. Phylogenetic tree showing partial (*Beaudette* GenBank accession number NP 04083 amino acid position 48 [nucleotide 144] to 219 [nucleotide 657]) S1 gene inter-relationships between IBV field isolates from Israel, selected reference strains and commercial H120 vaccines. * H120 Vaccine 1 used for immunization in the vaccination-challenge of immunity trial (Table 7).

Table 5. Protection of chickens following vaccination with IBV strains and challenged with US field isolates with high (>91%) S1 sequence (amino acid) similarity values to the vaccines

Immunizing vaccine strain ^a	US field isolate used for challenge ^b	S1 sequence identity between immunizing and challenge virus (%)	Percent protection (number of IBV-positive/total birds)
Arkansas DPI	MD/30/99	93	77 (3/13)
None	MD/30/99		0 (5/5)
Arkansas DPI	MD/183/99	93	100 (0/11)
None	MD/183/99		0 (5/5)
DE/072/92	DE/322/99	93	100 (0/10)
None	DE/322/99		0 (5/5)
DE/072/92	DE/406/99	93	55 (5/11)
None	DE/406/99		0 (5/5)
DE/072/92	PA/5173/98	89	80 (2/10)
None	PA/5173/98		0 (5/5)
Massachusetts	DE/51/99	99	83 (2/12)
None	DE/51/99		0 (5/5)
Arkansas DPI	Arkansas DPI	100	72 (3/11)
None	Arkansas DPI		0 (5/5)
DE/072/92	DE/072/92	98	100 (0/11)
None	DE/072/92		0 (5/5)
Massachusetts	Massachusetts 41	95	82 (2/11)
None	Massachusetts 41		0 (5/5)

^aTwo-week-old SPF Leghorn chickens vaccinated ocularly with live commercial vaccine strains Arkansas DPI, DE/072/92 or Massachusetts.

^bChallenge viruses ($10^{4.0}$ to $10^{4.5}$ EID₅₀ per chicken) given ocularly at 6 weeks of age. Tracheal swabbings collected 5 days after challenge for virus isolation attempts.

identity values of 65 to 67% to the H120 vaccine strain.

Discussion

S1 partial gene sequencing and RFLP analyses were used to determine the relationship of field isolates to vaccine strains, previously described reference strains or new variant genotypes. Field isolates from the US and Israel could be placed into four categories: isolates highly related to vaccines; isolates highly related to reference strains; isolates related to, yet distinguishable from, vaccines or reference strains; or variants unrelated to vaccines or reference strains.

Nine of 40 isolates in the study were highly related (>99%) by sequencing to Mass serotypes vaccines. US isolate DE/51/99 was most highly related to Mass vaccines used in the US (Figure 1). Eight Israeli isolates (IS/236/96, IS/361/97, IS/373/97, IS/388/97, IS/415/97, IS/617/98, IS/665/98, and IS/51355/98) were highly related to H120 vaccines used in Israel (Table 4 and Figure 2). IS/361/97 and IS/388/97 were isolated from broilers with renal lesions consistent with nephropathogenic IB. H120 vaccine may have persisted in the birds and was inadvertently recovered from the flock, but was not the cause of the renal disease. A pathogenesis study could establish the potential role of the isolates in the outbreak.

Isolates highly related to reference strains included IS/222/96, IS/251/96, and IS/64714/96 to Variant 1 ($\geq 98\%$ S1 identity) and IS/223/96, IS/572/98, IS/585/98, and IS/589/98 to Variant 2 (99% S1 identity). Variant 1 and Variant 2 viruses were first reported in Israel in 2001

(Callison *et al.*, 2001). Variant 1 viruses are members of the genotype that includes strains 4/91 and 7/93B from the UK (Gough *et al.*, 1992; Parsons *et al.*, 1992).

US isolates related to, yet distinguishable from, vaccines or reference strains were also identified (Table 3 and Figure 1). MD/30/99 and MD/183/99 were related to, yet distinguishable from, Ark DPI vaccine with S1 similarities of 94 and 93%, respectively. The isolates had the highest S1 sequence identity (98%) to reference strain Ark/1529/95, recovered from broilers from the Delmarva Peninsula region in 1995 (Table 3 and Figure 1). Ark/1529/95 was identified as a quasi-species in Ark DPI vaccines, and was thought to have reverted to virulence through back-passage (Nix *et al.*, 2000).

Isolates AL/2975/00, AL/3701/00, DE/322/99, DE/406/99, GA/5425/99 and PA/5173/98 from the Delmarva and Southeast regions of the US were also related to, yet distinguishable from, the DE/072/92 reference strain (S1 identity 89 to 92%) and the commercial vaccine (S1 identity 91 to 92%). The relatively low identity values to the vaccine suggest the field isolates originated from an unknown source in the field and were not derived from either the DE/072/92 reference strain or the commercial vaccine. Furthermore, the Delmarva and Southeast isolates were also different from each other with S1 similarities from 89 to 92% (data not shown). The identification of three related yet distinguishable DE/072/92 RFLP groups (A-1, A-2 and A-3) also suggested that the field isolates are similar but not identical. Our findings support initial (Gelb *et al.*, 1997) and later reports (Lee *et al.*, 2001) demonstrating the diversity within the DE/072/92 genotype. It appears that DE/072/92 genotypes are evolving from multiple origins in the field.

Table 6. Protection of chickens following vaccination with strains Massachusetts and Arkansas against challenge with variant US field isolates

Immunizing vaccine strains ^a	Challenge virus ^b	S1 sequence identity between immunizing and challenge viruses (%)		Percent protection (number of IBV-positive/total birds)
		Massachusetts	Arkansas	
Massachusetts and Arkansas	CA/510/99	69	83	75 (3/12)
None	CA/510/99			0 (5/5)
Massachusetts and Arkansas	PA/171/99	62	74	91 (1/12)
None	PA/171/99			20 (4/5)
Massachusetts and Arkansas	PA/5083/99	62	72	91 (1/12)
None	PA/5083/99			40 (3/5)
Massachusetts and Arkansas	PA/5344/98	67	72	50 (6/12)
None	PA/5344/98			0 (5/5)
Massachusetts and Arkansas	MN/113575/99	65	68	100 (0/12)
None	MN/113575/99			0 (5/5)
Massachusetts and Arkansas	Mass 41	95	64	82 (2/11)
None	Mass 41			0 (5/5)
Massachusetts and Arkansas	Ark DPI	66	99	91 (1/11)
None	Ark DPI			0 (5/5)

^aTwo-week-old SPF Leghorn chickens vaccinated ocularly with live vaccine strains Massachusetts and Arkansas.

^bChallenge viruses ($10^{4.0}$ to $10^{4.5}$ EID₅₀ per chicken) given ocularly at 6 weeks of age. Tracheal swabbings collected 5 days after challenge for virus isolation attempts.

Among Israeli isolates, two were related to, yet distinguishable from, the H120 vaccine by S1 sequence and RFLP analyses (Table 4 and Figure 2). Mass genotypes IS/287/96 and IS/385/97 were not highly related to H120 (95 and 96%, respectively), but were also not highly related to Mass 41 (92 and 94% identity, respectively). The isolates may represent indigenous Mass genotype strains circulating in Israeli poultry.

US isolates PA/4327/97 and PA/5083/99 were related to, yet distinguishable from, reference strain PA/Wolgemuth/98, one of two unique genotypes found during a nephropathogenic outbreak in Pennsylvania (Ziegler

et al., 2002). PA/4327/97 and PA/5083/99 had sequence identity values of 94 and 96%, respectively, compared with PA/Wolgemuth/98. S1 similarities of PA/4327/97, PA/5083/99, and PA/Wolgemuth/98 to the other genotype identified in the outbreak, PA/171/99, ranged from only 80 to 85% (Ziegler *et al.*, 2002). RFLP analysis supported the sequence findings indicating the PA/Wolgemuth/98 and PA/171/99 genotypes are different (Table 3).

US isolates CA/1249/99, MN/11375/99, and PA/1220/98 had unique S1 sequences that classified them as variants unrelated to vaccines or reference strains

Table 7. Relationship of protection and S1 sequence (amino acid) similarity values of IS field isolates and a commercial live IBV vaccine containing the H120 strain

Immunizing vaccine strain ^a	Challenge virus ^b	S1 sequence identity to H120 (%)	Percent protection (number of IBV-positive/total birds)
H120	IS/720/99	65	36 (7/11)
None	IS/720/99		0 (6/6)
H120	IS/64714/96 (Variant 1) ^c	66	58 (5/12)
None	IS/64714/96 (Variant 1)		16 (5/6)
H120	IS/585/98 (Variant 2) ^d	67	25 (9/12)
None	IS/585/98 (Variant 2)		0 (6/6)
H120	IS/385/97	96	58 (5/12)
None	IS/385/97		16 (5/6)
H120	IS/665/98	99	83 (2/12)
None	IS/665/98		0 (6/6)

^aTwo-week-old SPF Leghorn chickens vaccinated ocularly with live vaccine strain H120.

^bChallenge viruses ($10^{4.0}$ to $10^{4.5}$ EID₅₀ per chicken) given ocularly at 6 weeks of age. Tracheal swabbings collected 5 days after challenge for virus isolation attempts.

^cIsraeli IBV field isolate representative of the Variant 1 genotype.

^dIsraeli IBV field isolate representative of the Variant 2 genotype.

(Table 3 and Figure 1). Isolates CA/1249/99, MN/11375/99, and PA/1220/98 had low identity values to known genotypes. RFLP analysis also identified these isolates as having unique restriction patterns.

Isolate IS/720/99 was also classified as a variant (Table 4 and Figure 2). In Israel, isolates of the same genotype continue to cause IB but recent cases have been associated with renal disease (Meir *et al.*, 2004). In 2001, we identified another highly related isolate of the IS/720/99 genotype from Egypt (Egypt/Beni-Seuf/01; AF395531) (Abdel-Moneim *et al.*, 2002), associated with both renal and respiratory forms of the disease.

Three US isolates could not be classified as either related to a vaccine or reference strain or a variant by sequencing. S1 similarities for CA/510/99 (83% to Ark DPI), CA/3402/99 (83% to Ark DPI), and PA/5344/98 (85% to JMK) were higher than those typically associated with variants (Table 3). Until the year 2000, we considered partial S1 sequences of variant genotypes to be $\leq 75\%$ similar to a reference or vaccine strain (Kingham *et al.*, 2000). Since that time, we have encountered several isolates such as CA/510/99, CA/3402/99, and PA/5344/98 with S1 identity values up to 85%. Antigenic characterization using virus neutralization and reciprocal cross-challenge studies in chickens may provide biological relevance to "intermediate" S1 genetic inter-relationships.

In challenge of immunity trials, IBV vaccines with high S1 identity values ($\geq 93\%$) were protective using four of six field isolates (Tables 5 and 7). Other reports (Cavanagh *et al.*, 1997; Gelb *et al.*, 1997) using non-attenuated field strains for immunization have described similar results. Partial protection was observed against the remaining two field isolates. DE/072/92 immunization versus DE/406/99 challenge produced 55% protection, and H120 vaccination resulted in 58% protection, against IS/385/97. Hodgson *et al.* (2004) reported that a recombinant Beaudette with the S gene of Mass 41 induced better protection against challenge with Mass 41 than the original non-recombinant Beaudette strain even though the S1 proteins differed by only 5%. Factors such as differences in epitopes of the vaccine strains and challenge viruses used may have influenced the challenge findings. Moreover, protection results from challenge assays sometimes display a degree of variability that is a function of the relatively few chickens used.

The degree of *in vivo* cross-protection often declines with decreasing S1 sequence homology between isolates used for IBV immunization and challenge (Cavanagh *et al.*, 1997; Gelb *et al.*, 1997). H120 vaccination provided little protection versus challenge with IS/720/99 (36%) and Variant 2 (25%). This finding is of concern since H120 is the only licensed live vaccine strain in Israel.

In contrast, immunization with a heterologous vaccine strain(s) may cross-protect even when there are considerable differences in S1 similarities between the vaccine and challenge strains (Table 6). The phenomenon of IBV cross-protection has been recognized for many years (Hofstad, 1981; Cook *et al.*, 1999) and has been attributed to the immune response to shared S1 as well as other IBV gene epitopes (Cavanagh *et al.*, 1997). Immunization with a bivalent vaccine containing the Mass and Ark strains provided cross-protection against challenge with IBV field strains (Gelb *et al.*, 1991). Protection $\bar{x} = 81\%$, value not in Table 6) was observed

against challenge with CA/510/99, PA/171/99, PA/5083/99, PA/5344/98, and MN/113575/99. H120 vaccination provided partial protection (58%) against Variant 1 (Table 7). The findings illustrate the importance of evaluating the efficacy of vaccination against isolates that may be quite unique based on sequencing.

Discrepancies between RFLP and sequencing results occurred with US isolates DE/51/99, MD/183/99 and MD/30/99 (Table 3). DE/51/99 was identified as JMK by RFLP and as Mass by sequencing. RE analysis of available S1 sequences for DE/51/99 indicated that the discrepancy could be explained by a point mutation(s) at *Bst*YI and *Xcm*I restriction sites, which yielded two fragments instead of three. Sequencing findings identifying DE/51/99 as a Mass genotype were supported by challenge of immunity results (Table 5). MD/183/99 and MD/30/99 were identified as different genotypes (RFLP Group D and Group G, respectively). However, both isolates were highly related (98%) by sequencing to Ark/1529/95 (Table 3). RE analysis using available partial sequences for MD/183/99 and MD/30/99 were not helpful in resolving the discrepancy. Challenge of immunity results supported the sequencing findings that MD/183/99 and MD/30/99 were Ark genotypes (Table 5).

S1 sequence and RFLP analyses can be used to identify field strains related to vaccines and novel variants. Comparison and analysis of sequences of unknown field isolates with reference strains for establishing potential relatedness is an advantage of sequencing versus RFLP.

Although S1 gene-based diagnostics have made IBV identification faster, the challenge of disease control remains. Controlling IB is quite feasible when the causative field strain and an available vaccine strain share a high degree of sequence identity. However, many field isolates have low ($\leq 85\%$) S1 identity values or unique RFLP patterns compared with vaccines. In these situations, the options for controlling the disease may be limited. Selection of monovalent or bivalent vaccines known to induce heterologous cross-protection could yield improved performance. When possible it is important to periodically evaluate the cross-protective capabilities of vaccines versus recently recovered field isolates. Outcomes of these studies will provide valuable information on the practical use of existing vaccines and the potential need for new ones.

Acknowledgements

This research was supported by Research Grant No. US-3068-98 from BARD, the United States-Israel Binational Agricultural Research and Development Fund.

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Translations of the abstract in French, German and Spanish are available on the *Avian Pathology* website.

Non-English Abstracts

S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000)

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Caractéristiques du gène S1 et efficacité de la vaccination contre les souches de virus de la bronchite infectieuse isolées aux USA et en Israël (1996–2000)

Les gènes S1 des souches de coronavirus aviaire de la bronchite infectieuse (IBV) isolées chez des poulets de chair aux USA et en Israël (20 souches de chaque pays) ont été étudiées en utilisant les techniques de transcription inverse (RT), d'amplification en chaîne par polymérase (PCR), de polymorphisme de taille des fragments de restriction de l'acide nucléique (RFPL) et de séquençage. Les séquences partielles de la région aminée terminale du gène S1 comprise entre les résidus acides aminés 48 et 219, ont été analysées sur la base de la souche Beaudette. Les regroupements phylogénétiques et les valeurs élevées d'identité de séquence ont été utilisés pour identifier les isolats qui apparaissaient être dérivés des vaccins vivants utilisés dans les deux pays. Au vu des résultats du séquençage et des analyses de RFLP, de nouvelles souches variantes ont été identifiées, sans lien avec les souches de référence et les souches vaccinales. En se basant sur l'identité de la séquence de S1 des souches vaccinales disponibles, la possibilité d'utiliser la vaccination pour contrôler les infections à IBV a été évaluée. La vaccination avec des souches vivantes commerciales Massachusetts (Mass), Arkansas (Ark) ou DE/072/92, a généralement entraîné une immunité vis-à-vis des souches du terrain ayant un lien avec les souches vaccinales montrant des similarités élevées de la séquence de S1 ($\geq 90\%$) avec celles des souches vaccinales respectives. L'immunisation avec un vaccin bivalent contenant les souches Mass et Ark a conféré une bonne protection croisée, de 81% en moyenne, vis-à-vis d'épreuves avec cinq virus variants isolés aux USA ayant des niveaux d'identité en acides aminés allant de 62 à 69% vis-à-vis de la souche Mass et de 68 à 83% vis-à-vis de la souche Ark. Par contre, la souche vaccinale H120 a induit des taux faibles de protection, allant de 25 à 58% vis-à-vis des variants isolés en Israël ayant des niveaux d'identité en acides aminés allant de 65 à 67%.

Charakteristika des S1-Gens und Wirksamkeit der Vakzination gegen Feldisolate des Virus der infektiösen Bronchitis aus den USA und Israel (1996–2000)

Die S1-Gene von Isolaten des aviären Coronavirus infektiöse Bronchitisvirus (IBV) aus kommerziellen Hühnern in den USA und Israel (20 Isolate aus jedem Land) wurden mittels reverser Transkription (RT)-Polymerasekettenreaktion (PCR), Restriktionsfragmentlängen-polymorphismus (RFLP) und Sequenzierung untersucht. Sequenzstücke, die basierend auf dem Beaudette-Stamm, die Aminoendregion des S1 mit den Aminosäurenresiduen 48–219 umfassten, wurden für die Analyse verwendet. Phylogenetische Clusterung und hohe Sequenzübereinstimmungswerte dienten dazu, die Isolate zu identifizieren, die wahrscheinlich von in den beiden Ländern verwendeten Lebendvirusvakzinen abstammten. Neue Variantstämme, die aufgrund der S1-Sequenzierung und der RFLP-Analyse nicht mit den Referenz- und Vakzinestämmen verwandt waren, wurden auch nachgewiesen. Basierend auf der S1-Sequenzübereinstimmung der untersuchten Isolate mit den verfügbaren Vakzinen wurde die Eignung der Vakzination zur Bekämpfung von IBV-Infektionen ermittelt. Die Vakzination mit den kommerziellen Lebendvirusstämmen Massachusetts (Mass), Arkansas (Ark) oder DE/072/92 erzeugte im allgemeinen eine Immunität gegen Vakzine-verwandte Feldisolate mit hohen S1-Sequenzähnlichkeiten ($\geq 90\%$) zu den entsprechenden Vakzinestämmen. Die Immunisierung mit einer bivalenten Vakzine mit den Stämmen Mass und Ark erzeugte einen guten Kreuzschutz von im Mittel 81% gegen Belastungsinfektionen mit fünf Variantstämmen aus den USA, die in ihren Aminosäuresequenzen zu 62–69% mit Mass bzw. zu 68–83% mit Ark übereinstimmten. Im Gegensatz dazu

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Received 17 November 2004. Accepted February 2005

ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/05/30001-02 © 2005 Houghton Trust Ltd

DOI: 10.1080/03079450500096539

induzierte der Vakzinestamm H120 nur eine geringe Schutzwirkung von 25–58% gegen Variant-Feldisolate aus Israel, die zum Impfstamm Übereinstimmungswerte der Aminosäuren von 65–67% aufwiesen.

Características del gen S1 y eficacia de la vacunación frente a aislamientos de campo del virus de la bronquitis infecciosa de Estados Unidos e Israel (1996–2000)

Se estudió el gen S1 de aislamientos del coronavirus de la bronquitis infecciosa aviar (IBV) procedentes de pollos comerciales de Estados Unidos e Israel (20 aislamientos de cada país) mediante el uso de la transcripción reversa (RT), reacción en cadena de la polimerasa (PCR), polimorfismo de la longitud de los fragmentos de restricción y secuenciación. Se usaron para el análisis, secuencias parciales que abarcaban la región amino terminal de la S1 entre los residuos aminoacídicos 48–219, en base a la cepa Beaudette. El agrupamiento filogenético y los valores de mayor identidad de secuencias se usaron para identificar aislados que parecían proceder de vacunas vivas de IBV utilizadas en ambos países. También se identificaron nuevas cepas variantes, no relacionadas mediante secuenciación de la S1 y análisis de RLFP a las cepas vacunales y de referencia. Se evaluó la posibilidad de usar la vacunación en el control de las infecciones por IBV, basándose en la identidad de las secuencias de la S1 respecto las vacunas disponibles. La vacunación con cepas vivas comerciales Massachussets (Mass), Arkansas (Ark) o DE/072/92, generalmente produjo inmunidad frente a los aislamientos de campo relacionados con las vacunas, mostrando elevadas similitudes ($\geq 90\%$) de la secuencia de la S1 con las respectivas cepas vacunales. La inmunización con una vacuna bivalente que contiene las cepas Mass y Ark proporcionó buena protección cruzada con una media del 81% frente a la infección experimental con cinco aislamientos variantes de US con una identidad aminoacídica entre 62–69% frente a Mass y 68–83% frente a Ark, respectivamente. Por el contrario, la cepa vacunal H120 indujo niveles bajos de protección, oscilando entre el 25–58% frente a los aislamientos variantes de campo de Israel con identidades aminoacídicas entre el 65–67%.