

Cross-Protection Test of an Avian Poxvirus Isolated from Houbara Bustards

Stéphane Ostrowski,^{AD} Gerry Dorrestein,^B Louis Burger,^C
Stéphane Hémon,^A and Michel Saint Jalme^A

^ANational Wildlife Research Center,
National Commission for Wildlife Conservation and Development,
P.O. Box 1086, Taif, Saudi Arabia

^BDepartment of Veterinary Pathology, Section of Exotic Animals,
Veterinary Faculty of Utrecht, Yalelaan 1, De Uithof, 3584 CL Utrecht, The Netherlands

^CPoultry Health Institute, Oude Rijkssstraatweg 43,
Postbus 43, 3940 AA Doorn, The Netherlands

Received 9 August 1995

SUMMARY. An avian poxvirus was isolated previously from the houbara bustard (*Chlamydotis undulata*). We carried out a cross-protection test on 66 captive-bred canaries. Thirty-five canaries were vaccinated with a commercial canary poxvirus (CP) vaccine. Three weeks later all 66 birds were assigned randomly to six different groups: group Ia (n = 14) was vaccinated and challenged with houbara bustard poxvirus (HP) strain; group Ib (n = 13) was vaccinated and challenged with a CP strain; group Ic (n = 7) was vaccinated and not inoculated; group IIa (n = 14) was nonvaccinated and challenged with HP strain; group IIb (n = 11) was nonvaccinated and challenged with a CP strain; and group IIc (n = 7) was not vaccinated and not challenged. Vaccinated groups (Ia, Ib, Ic) had no losses and remained healthy. All of the birds (100%) in group IIb died within 10 days, and 10 birds (71.4%) of group IIa died within 20 days. The nonvaccinated control group (IIc) remained healthy. Poxvirus was isolated from the liver, digestive tract, lungs, and inoculation lesions of nonvaccinated dead CP- and HP-challenged birds. Secondary bacterial infections were higher among nonvaccinated HP-challenged birds (85.7%) than in nonvaccinated CP-challenged birds (25%). The results of this experiment reveal a degree of immunogenic relatedness between CP and HP strain and support the recommendation that houbara bustards be vaccinated with a CP vaccine.

RESUMEN. Prueba de protección cruzada de un virus de viruela aviar aislado de avutardas.

Un virus de viruela aviar fue aislado previamente de avutardas (*Chlamydotis undulata*). Nosotros realizamos una prueba de protección cruzada en 66 canarios criados en cautiverio. Treinta y cinco canarios fueron vacunados con una vacuna comercial de virus de viruela de canario. Tres semanas después, las 66 aves fueron asignadas al azar a 6 grupos diferentes: el grupo Ia (n = 14) fue vacunado y desafiado con una cepa de viruela de avutarda; el grupo Ib (n = 13) fue vacunado y desafiado con una cepa de viruela de canario; el grupo Ic (n = 7) fue vacunado y no inoculado; el grupo IIa (n = 14) no fue vacunado y se desafió con una cepa de viruela de avutarda; el grupo IIb (n = 11) no fue vacunado y se desafió con la cepa de virus de canario; y el grupo IIc (n = 7) no fue vacunado ni desafiado. En los grupos vacunados (Ia, Ib, Ic) no hubo pérdidas y todas las aves permanecieron sanas. Todas las aves (100%) del grupo IIb murieron en 10 días, y 10 aves (71.4%) del grupo IIa murieron en 20 días. El grupo control no vacunado (IIc) se mantuvo sano. El virus de viruela se aisló del hígado, tracto digestivo, pulmones y de las lesiones de inoculación de las aves muertas no vacunadas desafiadas con virus de canarios y avutardas. Las infecciones bacterianas secundarias fueron más altas en las aves no vacunadas desafiadas con virus de avutardas (85.7%) que en las aves no vacunadas desafiadas con el virus de canario (25%). Los resultados de este experimento revelan que existe un grado de relación inmunogénica entre las cepas de virus de

^DCorresponding author.



canario y avutarda, y justifica la recomendación de que las avutardas deben ser vacunadas con una vacuna de virus de viruela de canario.

Key words: Houbara bustard, poxvirus disease, canary poxvirus strain, cross-protection test

Abbreviations: CAM = chorioallantois membrane; CCID₅₀ = cell culture infectious dose 50%; CP = canary poxvirus; HP = houbara poxvirus; PI = postinoculation

Poxvirus infection in birds is a slowly spreading viral disease, inducing cutaneous lesions and/or diphtheric lesions in the digestive and respiratory tracts (17). Avian poxvirus infection has been reported from numerous species of birds and has been associated with morbidity and mortality in wild and domestic birds (12,14). The houbara bustard (*Chlamydotis undulata*) has been classified as "vulnerable" (taxa with populations that have been seriously depleted and whose ultimate security has not been assured) by the World Conservation Union (20). At the National Wildlife Research Center (Taif, Saudi Arabia), captive breeding of houbara bustards was initiated in 1986, with the aim of restoring wild populations. Avian poxvirus occurs in the breeding unit as a mild latent infection of high morbidity and low mortality. A debilitating case of cutaneous poxvirus disease has already been described in the houbara bustard and confirmed by virological isolation (15).

Preparing a safe vaccine with the bustard isolate would mean carrying out long titration studies in houbara bustards after every embryo passage. Houbara bustards are too valuable to use as subjects in direct pathogenicity experiments. The ideal situation would be to find an animal model that was sensitive to the houbara isolate and could be protected effectively by a commercially available vaccine. Agar-gel-immunoagglutination tests carried out on the houbara-isolated strain showed a positive reaction with canary poxvirus (CP) antibodies (15).

We carried out a cross-protection test on canaries to assess the pathogenicity of houbara poxvirus (HP) strain in this species and to determine whether a vaccination with a CP strain vaccine can protect these birds against an HP strain virus challenge.

MATERIALS AND METHODS

Viruses. A poxvirus was isolated from a cutaneous lesion involving the tibiotarso-tarsometatarsal joint of

a houbara bustard. Fresh excised tissues were frozen for virological examination. These samples and formalin-fixed material were shipped to the Department of Veterinary Pathology, Section of Exotic Animals, of the Veterinary Faculty of Utrecht, the Netherlands, for examination. The poxvirus was isolated from the samples by vortexing in 10 ml Hank's phosphate-buffered saline solution and centrifugation for 10 minutes at 3500 rpm. The supernatant was filtered through an 800 µm filter. The filtrate was propagated in chicken eggs. Seven days after infection, the chorioallantois membranes (CAM) were harvested and used for a second passage in chicken eggs.

The CAM were ground in a mortar with sterile sand and distilled water. The homogenate was clarified by centrifugation. It was used for an agar-gel-precipitation test against antibodies of chicken poxvirus and CP. This test was positive for CP. The homogenate was freeze-dried in lots of 1 ml and stored.

The canary vaccine (Kanapox, Rhone Mérieux, Lyon, France) was freshly prepared and used within 30 minutes after preparation. The inoculated dose was calculated to be 10³ cell culture infectious dose 50% (CCID₅₀) per dose. The vaccine titer was calculated by performing 10-to-10 dilutions of a viral suspension in Stocker media at 4 C until the dilution revealed 100% of infectivity. The virus suspension was diluted 4 to 4 until the dilution revealed 0% of infectivity. This suspension was inoculated to chicken embryo cellular cultures, incubated in an enriched carbon dioxide atmosphere for 7 days, and checked for cytopathogenic effect. The titer as log₁₀ of infectious cellular culture dose was expressed by 10³ CCID₅₀ per dose.

For the challenge experiments, the houbara strain (#16731) and a CP strain (GA-10657-4) were collected from the freezer, and one ampulla of each strain was titrated on the CAM of 10 day-incubated chicken eggs. The isolates were shipped freeze-dried to the National Wildlife Research Center.

The freeze-dried houbara and canary strains were reconstituted in 0.85% sterile saline. The final concentration for the HP strain (#NL-16731) was 10^{7.1} plaque-forming units per 0.5 ml, and for the CP strain (#GA-10657-4), 10^{6.5} plaque-forming units per 0.5 ml.

Experimental designs. Seventy adult canaries were obtained from a private breeder in Jeddah (Saudi Arabia) in February 1994. CP vaccination is not

routinely performed in this country, and none of the birds nor their parents had ever been vaccinated against poxvirus disease. Canaries were housed in four indoor flight cages ($2 \times 1.8 \times 1.2$ m) with a 9-hour light cycle at the Wildlife Research facility. The cages were crossed by 1-cm wooden perches. Birds were fed *ad libitum* from a perch feeder attached to the side of the cages and also from a 0.5–1 ground feeder. The diet consisted of two different canary maintenance chows (Trill, Waltham, and Allesterin canary mixture, Witte Mollen B.V.) mixed in a 1:1 ratio. Approximately 0.5 kg of fresh fruits supplemented the diet once a week. Mineral water was provided *ad libitum*, and a polyvitaminic complex (Vitapaulia/m, Distrivet, Paris, France) was added to the water every day. Birds were housed in the flight cages for 1 month before the experiments (February to March 1994).

During the vaccination and inoculation experiments, the canaries were housed in six separated cages ($90 \times 50 \times 70$ cm each). Wooden perches and *ad libitum* food and water were provided throughout each study. The cages were placed in pairs in isolated non-communicating rooms. Each room was air-conditioned, with a constant ambient temperature of 21 C.

The three groups were kept in strict confinement. Measures were taken to avoid any contact between them: different people fed them, and during feeding, handling, and manipulations, sterile gloves and overalls were used. Gloves were incinerated after use and overalls disinfected. Seed and water bowls were cleaned and disinfected daily by wiping off debris and soaking them in a 0.5% quaternary ammonium solution (Antec quaternary, Antec International) for 30 minutes.

Sixty-seven canaries of mixed gender were divided randomly into two groups. The birds were allowed to acclimatize to their new cages and social environment for 1 week. Then the birds of group I ($n = 35$) were vaccinated against poxvirus disease with a CP vaccine. The canaries of group II ($n = 32$) were inoculated with the solvent only. All inoculations were done through the wing web. Special attention was paid not to disrupt any blood vessel. Before inoculation, the wing web was disinfected with 70% ethylic alcohol, and the vaccinostyle was systematically disinfected and dried between inoculations.

All birds were checked daily. The vaccinated canaries were inspected for typical poxvirus lesions at the inoculation site at days 7, 10, and 13 postinoculation (PI). The nonvaccinated birds were inspected for lesions in the wing web at day 10 PI. Twenty-four days after vaccination, the canaries were assigned randomly to six different groups in the inoculation experiment:

Group Ia ($n = 14$), vaccinated and to be challenged with an HP strain.

Group Ib ($n = 13$), vaccinated and to be challenged with a CP strain.

Group Ic ($n = 7$), vaccinated controls.

Group IIa ($n = 14$), nonvaccinated and to be challenged with an HP strain.

Group IIb ($n = 11$), nonvaccinated and to be challenged with a CP strain.

Group IIc ($n = 7$) nonvaccinated controls.

The a, b, and c groups were housed each in a separate room in two adjoining cages. Birds of groups Ia and IIa were inoculated with 10 μ l HP strain, and birds of groups Ib and IIb with 10 μ l CP strain, through the wing web, after disinfection as described above. Particular efforts were made not to contaminate other parts of the bird and its direct environment. The control birds (Ic and IIc) were inoculated with 0.85% sterile saline.

All birds were checked daily. On days 6, 12, and 20 PI, all birds were examined and their body weights measured (± 0.1 g). Final body weight data were collected on the day of necropsy. Clinical examination included an inspection of the oral mucous membranes for diphtheric lesions and the wing web for local lesions. The local reaction to inoculation was evaluated using a lesion score from 1 to 6 (1 = no lesions; 2 = mild lesions with slight thickening and light inflammatory process; 3 = moderate lesions with noticeable cellular reaction and edema; 4 = severe lesions with significant cellular reaction, congestion, and extending edema; 5 = very severe lesions with extensive cellular reaction, acute inflammatory signs, and superficial ulcers; and 6 = severe local reaction with general symptoms). Diameters of local reactions (thickening of patagial membrane) were measured with callipers of minimal reading range, 0.1 mm. Local reactions of less than 0.1 cm were not considered specific to the inoculation but rather a normal healing reaction to wing-web perforation.

At the end of the experiment, canaries that did not die were kept in quarantine for 6 months and then were given to a private aviary in Jeddah.

Histology. All dead birds were necropsied. Post-mortem examinations were performed within 2 hours following death. Specimens were collected from various organs of HP- and CP-challenged dead birds, fixed in 10% neutral-buffered formalin, processed according to standard techniques, and stained with hematoxylin and eosin.

Bacteriology. Within 2 hours following death, saline-moistened swabs were used to collect samples from trachea, lungs, and local inoculation lesions of eight nonvaccinated CP-challenged and seven nonvaccinated HP-challenged dead birds. They were inoculated within 1 hour onto blood agar, MacConkey agar, and eosin–methylene blue agar media and incubated aerobically at 37 C for 72 hours. A quantitative evaluation of gram- bacteria invasion level of different tissues sampled was made on eosin–methyl-

line blue agar media after 72 hours of incubation. Colonies were counted and three categories were arbitrarily distinguished; 0-5, 6-15, and > 15 colonies. Bacteria were identified on the basis of growth characteristics on various media and biochemical profiles.

Virology. Poxvirus isolations were attempted from liver, digestive tract, lungs, and inoculation lesions. Samples submitted for virology were added to a buffered lactose peptone solution (pH = 7.4) containing penicillin (100 IU/ml) and streptomycin (100 mg/ml). They were frozen at -70 C and shipped to the Netherlands. Samples were inoculated into the CAM of 10-day-old embryonated chicken eggs. CAM were checked for typical focal pock lesions.

Statistics. All statistical calculations were performed using a χ^2 analysis with Yates correction. Serial recordings were compared by a Wilcoxon signed rank test for paired data and Mann-Whitney test (1).

RESULTS

Vaccination. Seven days after vaccination, 21 of the 35 birds presented a moderate primary vaccinal reaction, 2 showed a severe vaccinal reaction, and 11 had no reaction. One bird died accidentally 4 days after manipulation, and necropsy revealed a cervical trauma. Ten days after vaccination, 28 birds showed moderate primary lesions, 5 a severe reaction, and 1 no reaction. Finally, 13 days after vaccination, 25 birds still showed moderate primary reaction, 1 a severe reaction, and 8 no reaction. Vaccinal reaction was considered effective on 33 of 34 birds. One bird presented no vaccinal reaction 7, 10, and 13 days after vaccination. It was included in the challenge trial and considered as vaccinated. No significant vaccinal reactions were observed on the 32 birds inoculated with the solvent.

Challenges. Table 1 summarizes the results of challenges. Vaccinated birds challenged with the HP strain presented mainly mild to moderate lesions, although two presented a severe reaction. Twenty days after inoculation, 12 of them (85.7%) presented no lesions, and 2 still had mild lesions. Forty-five days after challenge, all of these birds were healthy, and their body weights were not significantly different from their weights at the day of inoculation (Wilcoxon test).

Vaccinated birds challenged with the CP strain presented mild to moderate lesions, except for one individual that presented a severe

Table 1. Summary of the results of canary poxvirus (CP) strain and houbara poxvirus (HP) strain challenge trials on nonvaccinated (group I) and vaccinated (group II) canaries.

Day	Nonvaccinated flock		Vaccinated flock		Vaccinated control flock	
	challenged with HP strain	challenged with CP strain	challenged with HP strain	challenged with CP strain	Nonvaccinated control flock	Vaccinated control flock
Mean lesion score ^a		3)	2.1 (n = 14)	2.5 (n = 12)	Nonvaccinated control flock	1.3 (n = 7)
			1.5 (n = 14)	2.1 (n = 12)		1.1 (n = 7)
			1.1 (n = 14)	1.3 (n = 12)		1 (n = 7)
Mean measurements of inoculation lesions (c.m)		3)	0.26 (n = 14)	0.25 (n = 12)		0.03 (n = 7)
			0.14 (n = 14)	0.16 (n = 12)		0.03 (n = 7)
			0.02 (n = 14)	0.02 (n = 12)		0.00 (n = 7)

^aLesion score: 1 = no lesions; 2 = mild lesions with slight thickening and light inflammatory process; 3 = moderate lesions with noticeable cellular reaction and edema; 4 = severe lesions with significant cellular reaction; 5 = very severe lesions with extensive cellular reaction; 6 = severe local reaction with general symptoms.

lesion with swelling and significant skin thickening 6 and 12 days after challenge. At 12 days after challenge, lesions in eight birds (66.6%) had healed or had never existed. Four of them still presented mild lesions with slight skin thickening. Forty-five days after challenge, all of these birds were healthy and had no visible lesions related to inoculation. Their body weights were not significantly different from their weights at the day of inoculation. One vaccinated canary challenged with the CP strain died on the day of inoculation. Necropsy and histology revealed severe enteritis, with coccidiosis associated with a severe hepatitis, and with necrosis suggestive of an atoxoplasma infection. Attempts to isolate the poxvirus from the brain, liver, and digestive tract remained unsuccessful. No significant differences in the size of lesions after inoculation in CP and HP strain-challenged vaccinated groups were noticed between 6 and 12 days after inoculation (Wilcoxon test). A significant difference (Mann-Whitney test, $P < 0.01$) in the size of lesions was noticed 6 days PI between the control group and HP strain-challenged birds and between the control group and CP strain-challenged birds. The difference was no longer significant between vaccinated challenged and control groups 12 and 20 days PI.

Vaccinated birds inoculated with 0.85% normal saline (Ic) did not present significant reaction (Table 1). Two of them had a nonspecific mild lesion measuring less than 0.1 cm 6 days PI, but this lesion disappeared 12 days PI.

Nonvaccinated canaries challenged with the HP strain (IIa) presented moderate to very severe lesions 12 days PI: seven birds (50%) had very severe local inflammation, superficial ulcers of the inoculation lesion, and discrete signs of respiratory distress. Within 12 and 20 days PI, 10 (71.4%) of them died: one at 13 days, three at 14 days, two at 15 days, two at 16 days, and two at 19 days after challenge. In addition to a very severe local lesion, all of them presented respiratory distress symptoms, poor appetite, and ruffled feathers. Sinusitis, conjunctivitis, and ocular discharge were also observed on four of them. Twenty days PI, the three remaining birds presented severe local lesions with fibrosis of the wing-web membrane. One had a moderate lesion associated with noisy respiration, sinusitis, and blepharitis.

Eight (72.7%) of the nonvaccinated birds challenged with the CP strain (IIb) died within 6 days of inoculation and did not develop a severe inoculation reaction. Two died at 4 days, three at 5 days, and three at 6 days after challenge. The three remaining canaries presented very severe lesions at the point of inoculation, with deep inflammation of the patagial membrane, signs of respiratory distress, and pseudomembranous lesions on the mucous membranes of buccal cavity. Two of them died 7 days and the last one 10 days PI.

Nonvaccinated canaries inoculated with 0.85% normal saline (Iic) did not present significant reactions (Table 1), although one had a mild nonspecific lesion (< 0.1 cm) 6 and 12 days PI.

No significant lesions were observed in the nonvaccinated control group between inoculation time, 6, 12, and 20 days. A significant ($P < 0.01$) increase in the size of the inoculation lesion was observed between 6 and 12 days after challenge in the nonvaccinated HP-challenged group (IIa). This significant difference was confirmed ($P < 0.001$) 6 and 12 days after challenge between the nonvaccinated HP-challenged group and the nonvaccinated control group. A comparison between nonvaccinated ($n = 14$) and vaccinated flocks ($n = 14$) challenged with the same HP strain revealed that local reaction to inoculation was not significantly different (Mann-Whitney test, $P = 0.16$) after 6 days but became highly significant ($P < 0.0001$) after 12 days. A comparison of mortalities within different groups showed that mortality in nonvaccinated CP- and HP-challenged groups was higher than in challenged vaccinated groups ($\chi^2 = 12.6$, $df = 1$, $P < 0.001$ between HP strain-challenged groups, and $\chi^2 = 16.8$, $df = 1$, $P < 0.001$ between CP strain-challenged groups).

Postmortem examinations of nonvaccinated HP- and CP-challenged birds confirmed the severity of local lesions. The swellings were up to 1 cm in diameter, smooth, congestive, and frequently ulcerated. The skin lesion in cross-section showed extending necrotic areas. Necrosis was a permanent feature in the swellings. Five HP-challenged canaries presented pseudomembranous lesions in the buccal cavity, pharynx, and trachea. Congestion of sinusal and nasal mucous membranes was systematically noticed

on nonvaccinated challenged birds. Three dead birds presented signs of steatosis of the liver.

Histology confirmed the poxvirus disease in HP- and CP-challenged dead birds. In HP-challenged dead birds, most of the lesions concerned the inoculation point, the respiratory tract, and the buccal mucous membranes. The patagium showed extensive bacterial invasions with exudative inflammation. In the skin, poxvirus lesions were observed, with small areas of proliferation of the stratum spinosum and epithelial cells containing eosinophilic cytoplasmic inclusions (Bollinger bodies). In some canaries, the buccal mucous membranes showed proliferation of the epithelium. Many cells contained poxviruslike inclusion bodies. Proliferation of bronchial epithelium with eosinophilic inclusion bodies were observed, and lesions diagnostic of poxvirus disease were seen in the lungs. These lesions were frequently associated with severe bacterial complications and reactive inflammation. In two birds, a bacterial infection of the respiratory tract associated with deep inflammation of bronchia, lungs, and air sacs—but no poxvirus lesions—were observed. Histological changes were rarely observed in other organs. The liver showed a mononuclear reaction in four canaries, and the trachea a chronic mononuclear infiltration in one bird. Mean body weights were noticeably lower in dead nonvaccinated HP-inoculated birds (mean = 15.37 g, SD = 2.20, n = 10) than in vaccinated birds (mean = 20.5 g, SD = 1.54, n = 14). Body weights were only slightly lower in nonvaccinated CP-inoculated birds (mean = 18.91 g, SD = 1.82, n = 11) compared with vaccinated birds (mean = 20.1 g, SD = 1.66, n = 13).

Microbiological isolations confirmed a bacterial invasion of inoculation lesions, trachea, and lungs of nonvaccinated HP- and CP-inoculated birds. Identified bacteria were mostly aerobic gram- bacilli. *Enterobacter* spp., *Acinetobacter* spp., and *Citrobacter freundii* were identified. Comparison of bacteriological invasion of nonvaccinated CP- and HP-inoculated birds showed that the number of colonies isolated from lungs, trachea, and inoculation lesions was always higher in HP-challenged birds than in CP-challenged ones. Virological studies were carried out on the lungs, liver, and digestive tract of 7 of 10 dead HP strain-challenged birds. Poxvirus was isolated from sampled or-

gans of two birds dead at 14 days and one bird dead at 19 days (42.8% of positive isolation). Virological studies were carried out on the same organs of 8 of 11 dead CP strain-challenged birds. Poxvirus was isolated from sampled organs of one bird dead at 4 days, two birds dead at 5 days, two birds dead at 6 days, and one bird dead at 7 days (75% of positive isolation). Virological work was not carried out on the canary dead 10 days after inoculation.

DISCUSSION

A number of workers (2,9) have reported that the development of the primary vaccinal reaction is important in the production of poxvirus immunity. The development of vaccinal reaction following the wing-web route of immunization in 33 of 34 (97%) of the vaccinated canaries suggested that the vaccine was potent. However, since a lack of primary vaccinal reaction does not necessarily indicate a lack of immunity response (19), all of the birds were included in the challenge trial.

Resistance to CP strain challenge among vaccinated birds was 100%, and mortality among nonvaccinated CP-challenged birds was total, demonstrating an excellent vaccinal protection. The CP incubation period established by experimental inoculation is 3 to 16 days, depending on the route of inoculation (4,6). Canary poxvirus is characterized by high mortality and a course of 3 to 10 days (6). Eight of the nonvaccinated canaries challenged with the CP strain died within 6 days after wing-web inoculation, which confirmed the high degree of virulence of the CP strain used. The illness was acute and generalized, although local inoculation lesion remained modest in size and inflammatory development.

On the contrary, nonvaccinated canaries inoculated with the HP strain showed a marked inoculation reaction 12 days after challenge. Mortality was observed only after 12 days, and the survival rate after 45 days was 21.4%, compared with 0% in nonvaccinated CP-inoculated birds.

Local inoculation lesions in nonvaccinated HP strain-inoculated birds were severe and always associated with extensive bacterial invasions. Although the bacterial contamination was higher in houbara poxvirus-induced lesions, it could result from a longer survival of

HP-inoculated birds, and therefore a greater possibility for secondary bacterial infection. Most frequently, isolated bacteria belonged to the *Enterobacteriaceae* family. A majority of the clinically healthy *Fringillidae* do not carry *Enterobacteriaceae* in their digestive (5) and respiratory (7b) tracts. Therefore, isolation of bacteria belonging to this family from the lungs, trachea, and inoculation lesions of challenged dead canaries supports the hypothesis of a combined infection. This group of bacteria acts frequently as secondary invaders. Although the genus *Enterobacter* is generally low in pathogenicity (7b), the genus *Citrobacter* is found in weavers, finches, and waxbills as a secondary invader of high pathogenicity (7b). In nonvaccinated HP strain-inoculated canaries, the heavy bacterial infection caused reactive inflammations in the respiratory tract (pneumonia), skin, buccal mucous membrane (ulceration), liver, and spleen.

Although the HP strain virus concentration inoculated into nonvaccinated canaries was higher than the CP strain concentration inoculated into nonvaccinated canaries, the latter proved to be more lethal. This quick mortality explains why body weights of dead nonvaccinated CP strain-inoculated birds were only slightly lower than body weights of dead nonvaccinated HP strain-inoculated birds. Mortality of CP strain-inoculated birds seemed to be linked directly to the virulence of the poxvirus itself, as very few bacterial complications were observed. In contrast, histology and bacteriology confirmed that secondary bacterial infections were predominant in the nonvaccinated HP strain-challenged group and could have played a role in the mortality. The importance of the inoculated HP as a primary pathogen factor is confirmed by the fact that vaccinated HP strain-inoculated birds kept in the same environmental conditions in adjoining cages and supporting the same bacteriological pressure did not present pathogen bacterial invasion.

The success of the present cross-protection test in CP-immunized birds suggests that there exists an immunological identity between CP and HP. These findings are in agreement with the reported relationship between CP and HP by agar-gel-precipitation tests (15). Nevertheless, results from serological studies and the present cross-protection test to assess the degree

of relatedness between the viruses are insufficient. Common antigens have been demonstrated among viruses of the avian pox group by complement fixation (16), viral neutralization (3), and agar-gel diffusion (18). The common antigens prevent these tests from being precise enough for identification of individual viruses of the avian poxvirus group. Understanding of the antigenic relatedness of HP and CP could have been improved by a reciprocal experiment in which canaries are immunized with a nonlethal dose of HP and then challenged with CP.

The present method gives only poor information on the specificity of the HP-strain virus in the canary. Usually, typical poxvirus lesions are produced in one species and only mild local reactions without inclusion-body in other species (8). In the present study, HP strain produced a severe local reaction with inclusion-body formation in canaries. Irons, 1934 (11), and Kirmse, 1969 (13), have exemplified the inadequacy of cross-pathogenicity tests to classify the strains. Because the HP strain was virulent for canaries, and CP is one of the four traditional classes of avian poxviruses, we can classify this virus as belonging to the CP class, recognizing the inadequacy of this system of nomenclature.

The evidence indicates that the HP strain is pathogenic for canaries and, when combined with a secondary bacterial infection, can lead to death in challenged canaries. However, the CP vaccine was suitable since it induced good immunity to virulent HP challenge when administered by wing-web stab. Observations within the houbara bustard facilities of the National Wildlife Research Center showed that pox disease among birds was epidemiologically linked to the presence of poxvirus-infected sparrows (10). Sparrows and canaries were highly susceptible to a poxvirus isolated during an outbreak from sparrows (8). Genomic characterization by restriction endonuclease analysis of DNA and antigenic characterization of immunogenic proteins by immunoblotting of HP are in progress and will identify the immunogenic and antigenic relatedness between canary, sparrow, and houbara poxvirus strains.

The results from this experiment support the recommendation that houbara bustard should be vaccinated with a CP vaccine. Subsequently, all of the birds in the breeding unit were vac-

inated, at a dosage of 2.10^3 CCID₅₀ per dose, by perforation of the wing web. This procedure resulted in a radical decrease in morbidity and mortality due to pox disease.

REFERENCES

1. Anonymous. Statgraphics version 4:0 Tutorial. STSC Inc., Rockville, Md. 1989.
2. Beaudette, F. R. Twenty years of progress in immunization against virus diseases of birds. *J. Am. Vet. Med. Assoc.* 115:232-244. 1949.
3. Burnet, F. M., and D. Lush. The immunological relationship between Kikuth's canary virus and fowl-pox. *Br. J. Exp. Pathol.* 17:302-307. 1936.
4. Cavill, J. P. Viral diseases. In: *Diseases of cage and aviary birds*. M. Petrak, ed. Lea & Febiger, Philadelphia, Pa. 1969.
5. Drewes, L. A., and K. Flammer. Clinical microbiology. In: *Clinical avian medicine and surgery*. G. J. Harrison and L. R. Harrison, eds. W. B. Saunders Company, Philadelphia, Pa. pp. 157-171. 1986.
6. Gerlach, H. Virus diseases in pet birds. *Proc. Ann. Meet. Assoc. Avian Vet.* pp. 87-109. 1983.
- 7a. Gerlach, H. Viral diseases. In: *Clinical avian medicine and surgery*. G. J. Harrison and L. R. Harrison, eds. W. B. Saunders Company, Philadelphia, Pa. pp. 408-433. 1986.
- 7b. Gerlach, H. Bacterial diseases. In: *Clinical avian medicine and surgery*. G. J. Harrison and L. R. Harrison, eds. W. B. Saunders Company, Philadelphia, Pa. pp. 434-453. 1986.
8. Giddens, W. E., L. J. Swago, J. D. Handerson, Jr., R. A. Lewis, D. S. Farner, A. Carlos, and W. C. Dolowy. Canary pox in sparrows and canaries (*Fringillidae*) and in weavers (*Ploceidae*). *Vet. Pathol.* 8: 260-280. 1971.
9. Graham, R., and C. A. Brandly. Immunization against pox in domestic fowl. *Univ. Illinois Agr. Exp. Stat. Bull.* 470:1-76. 1940.
10. Greth, A., H. Gerlach, B. Andral, and M. Vassart. Pathology of houbara bustards (*Chlamydotis undulata*) in captive breeding scheme in Saudi Arabia. Sixth International Conference on Wildlife Disease, August 6-11, Berlin, GDR. 1990.
11. Irons, V. Cross-species transmission studies with different strains of bird-pox. *Am. J. Hyg.* 20: 329-351. 1934.
12. Karstad, L. Pox. In: *Infectious and parasitic diseases of wild birds*. J. W. Davis, R. C. Anderson, L. Karstad, and D. O. Trainer, eds. Iowa State University Press, Ames, Iowa. pp. 34-41. 1971.
13. Kirmse, P. Host specificity and pathogenicity of pox viruses from wild birds. *Bull. Wildl. Dis. Assoc.* 5:376-386. 1969.
14. Kirmse, P. Pox in wild birds: An annotated bibliography. *Wildl. Dis.* 49:1-10. 1967.
15. Ostrowski, S., G. M. Dorrestein, M. Ancrenaz, and M. Saint Jalme. Debilitating cutaneous poxvirus lesion on two captive houbara bustards (*Chlamydotis undulata*). *Avian Dis.* Auepted. 1995.
16. Sato, T., T. Sugimori, S. Ishii, and M. Matumato. Etiologic study of an outbreak of canary pox in Japan 1958. *Jpn. J. Exp. Med.* 32:247-261. 1962.
17. Tripathy, D. N., and C. H. Cunningham. Avian pox. In: *Diseases of poultry*, 8th ed. M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder, eds. Iowa State University Press, Ames, Iowa. pp. 524-534. 1984.
18. Tsubahara, H., T. Kataoka, H. Nakamura, and H. Kawamura. Complement fixation in fowl pox. I. Complement fixation with infected tissues and pigeon serum. *Bull. Natl. Inst. Animal Health* 31: 48-56. 1956.
19. Winterfield, R. W., and S. B. Hitchner. The response of chickens to vaccination with different concentrations of pigeon pox and fowl pox viruses. *Avian Dis.* 9:237-241. 1965.
20. World Conservation Monitoring Centre. IUCN Red List of threatened animals, IUCN Publications Services Unit, Cambridge, England. 1992.

ACKNOWLEDGMENTS

The authors thank the National Commission for Wildlife Conservation and Development of Saudi Arabia, its Managing Director, H.R.H. Prince Saud Al Faisal, and Secretary General Prof. A. H. Abuzinada for their support of the project. Special thanks to Jacques Renaud, General Manager of the National Wildlife Research Center. The authors thank Abdul Rahman Khoja for smoothing the administrative path. The authors also thank M. Ancrenaz, D.V.M., and Dr. Y. van Heezik for their useful comments on the manuscript.