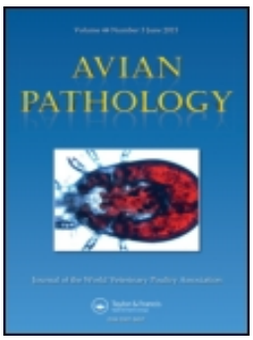

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Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera

A. P. Muhairwa*, J. P. Christensen and M. Bisgaard

Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Stigbøjlen 4, DK-1870 Frederiksberg C, Copenhagen, Denmark

Twenty flocks of web-footed birds (Pekin and Muscovy ducks and geese) and eight flocks of chickens raised under intensive management were examined for the presence of carriers of *Pasteurella multocida*. Five hundred and seventy-eight web-footed birds and 240 chickens from healthy flocks, as well as from flocks affected by fowl cholera, were investigated. A total of 135 isolates (80 from healthy flocks and 55 from flocks affected by fowl cholera) were obtained from the pharyngeal and cloacal mucosae after mouse passage (134 isolates) and culture in selective medium (one isolate). Thirty-five percent (7/20) of the flocks of web-footed birds and 38% (3/8) of chicken flocks had birds carrying *P. multocida* in the pharynx and/or cloaca. Birds from flocks affected by fowl cholera carried *P. multocida* at a significantly higher prevalence in the mucosa of the cloaca ($P < 0.001$) compared with the pharynx, while the opposite was observed in birds from healthy flocks. Extended phenotypic characterization confirmed the presence of *P. multocida* ssp. *multocida, P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida* in the flocks examined. *P. multocida* ssp. *gallicida* was exclusively isolated from Pekin ducks, while *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* were obtained from chickens as well as web-footed birds. Each flock was shown to be infected by a single phenotypic clone, but some clones were found in more than one flock. A different clone was found in each of four outbreaks of fowl cholera on one of the farms in the preceding 2 years. Two genotypic and phenotypic clones each of *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* were found. This observation indicated that outbreaks are usually clonal and that elimination of *P. multocida* from infected farms is possible. The results suggest that healthy poultry, in addition to convalescent carriers, may also be carriers of *P. multocida*. However, the virulence of *P. multocida* isolates and resistance of carriers to clinical infection needs to be examined. This is the first report of isolation of *P. multocida* from the cloacal mucosa of apparently healthy domestic poultry. Sampling of the cloaca appeared to be more sensitive for detecting carriers of *P. multocida*. Although selective medium was used only to a limited extent, the results suggested that mouse inoculation was a more efficient method of isolating *P. multocida* from poultry than the use of selective media.

Introduction

Despite considerable research on fowl cholera in domestic and wild birds, the source of new infections in the flocks is still uncertain. *Pasteurella multocida* has been consistently found in the upper respiratory tract, spleen, lungs, blood and liver of infected birds (Rhoades, 1964; Hunter & Wobeser, 1980). Studies in chickens and turkeys found *P. multocida* in the mucosae of the pharynx and trachea of carrier chickens and turkeys (Mraz *et al.*, 1980; Curtis & Ollerhead, 1981). Little has been done to investigate *P. multocida* in sites other than the upper respiratory tract in healthy carrier chickens since Iliev *et al.* (1964) failed to detect viable *P. multocida* in the faeces of chickens. However,

*To whom correspondence should be addressed. Tel: +45 3528 2749. Fax: +45 3528 2757. E-mail: apm@kvl.dk
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Bisgaard (1995) isolated *P. multocida* ssp. *multocida* from the oviduct of ducks and geese suffering from salpingitis, and also isolated *P. multocida* ssp. *multocida* from the cloacae and penises of geese from a flock with mortalities due to salpingitis and peritonitis.

Comparative studies of flocks of chickens and turkeys either exposed or unexposed to *P. multocida* by Curtis & Ollerhead (1981), Snipes *et al.* (1988) and Carpenter *et al.* (1989) revealed that *P. multocida* carriers were present only in flocks that had previously experienced fowl cholera. In a longitudinal study, Pritchett *et al.* (1930) isolated the bacterium for up to 4 months after a fowl cholera outbreak in a chicken flock. However, it is not known if the same clone was involved because reliable methods for investigating clonality did not exist at that time. Sulong & Maheswaran (1976) detected *P. multocida* by immunofluorescence in tissues of turkeys vaccinated with a virulent strain for up to 3 weeks after vaccination. All these studies suggest that some birds remain carriers of *P. multocida* for some time after exposure.

The recovery of *P. multocida* from different hosts has mainly relied on mouse inoculation and *in vitro* culture in selective medium (Curtis & Ollerhead, 1981; Baldrias *et al.*, 1988). Mouse passage seems to represent a more efficient and widely accepted method of detecting *P. multocida*, although it may only select strains pathogenic for mice (Baldrias *et al.*, 1988). Selective media have been devised for different host species of *P. multocida*, but efficacies of these media have not been compared (Rimler & Rhoades, 1989). Moore *et al.* (1994) demonstrated that selective enrichment coupled with blood agar inoculation have potential for increasing the isolation rate of *P. multocida* from contaminated samples. However, the necessity of employing two media might be a disadvantage in large-scale epidemiological studies. Serological techniques for detection of carriers of *P. multocida* appear to be of little use, as non-carriers may have antibodies to *P. multocida* (Rimler & Rhoades, 1989; Donnio *et al.*, 1994). Furthermore, with the ongoing reclassification of the genus *Pasteurella* (Mutters *et al.*, 1985), application of serological studies to identify the exact species or subspecies of *Pasteurella* might be more difficult.

Serological and biochemical characterization of isolates have been widely applied in epidemiological typing of *P. multocida* (Donahue & Olson, 1972; Heddleston *et al.*, 1972). However, in the past decade, it has been shown that chromosomal DNA studies are more discriminatory than phenotypic features in establishing the clonality of strains of *P. multocida* (Wilson *et al.*, 1995; Christensen *et al.*, 1998). Recent investigations of two outbreaks of fowl cholera in birds in Denmark demonstrated that the two outbreaks which appeared at different localities were caused by the same clone, strongly suggesting spread by migratory birds (Christensen *et al.*, 1998). Subsequent investigations indicated that wild birds can also transmit infection to domestic birds (Christensen *et al.*, 1999). Although cats and dogs are usually asymptomatic carriers (Baldrias *et al.*, 1988; Ganiere *et al.*, 1993), their significance in the epidemiology of fowl cholera remains to be confirmed.

It was the objective of the present work to determine the carrier rate of *P. multocida* in flocks of ducks, geese and chickens that had a confirmed history of fowl cholera and in flocks that had never experienced disease. Isolates were characterized phenotypically and genotypically to look for clonal relationships between strains, within and between the flocks.

Materials and Methods

Study population

Except for a single flock of Muscovy ducks, in which only eight ducks were sampled, 30 birds were sampled from each flock to allow a detection level of 10% with 95% certainty (Cannon & Roe, 1982). A total of 578 web-footed birds from 20 flocks and 240 chickens from eight flocks were sampled. Healthy birds and flocks investigated included three parent flocks of Pekin ducks from different farms, seven flocks of parent Pekin ducks from different houses on the same farm with history of bacteriologically confirmed fowl cholera in the previous flocks, and three independent flocks of Muscovy ducks (one flock of parents and two fattening flocks). Other healthy flocks sampled were two parent flocks of geese representing different farms, five fattening flocks of Pekin ducks originating from five different farms, four broiler parents flocks (one flock vaccinated against fowl cholera with the killed vaccine (Pabac, Fort Dodge Laboratories, Iowa, USA)) and two flocks of layers, all of which originated from different farms. One flock of broiler parents and one flock of brown layers investigated were reported to have mortalities caused by fowl cholera at the time of sampling. With the exception of seven flocks of parent Pekin ducks, healthy flocks were randomly selected and came from different geographical areas.

Isolation and bacteriological investigations

The birds were swabbed separately with sterile cotton-tipped applicators on the pharynx and cloaca. From nine flocks, each swab was streaked on a selective medium (Tryptose blood agar base (Difco) to which 5% citrated bovine blood, 0.02% bacitracin and 1% neomycin were added). The swabs were subsequently placed in 2 ml tryptophan broth (Difco) or veal infusion broth (Difco) and kept on ice during transport. The swabs were subsequently vortexed and 0.25 ml of the material injected intraperitoneally into 21-day-old Balb C white mice. From 19 flocks, attempts to isolate *P. multocida* included only inoculation of mice. In two fattening flocks of Muscovy ducks, the swabs from cloaca and pharynx were mixed before mouse inoculation.

Spleens from the dead mice were aseptically removed and macerated under sterile conditions, and the material cultured on blood agar without antibiotics. After overnight incubation at 37C under aerobic conditions, colonies morphologically resembling those of *P. multocida* and containing non-motile Gram-negative rods, which were facultatively anaerobic, fermented glucose without gas formation, and catalase- and oxidase-positive, were subcultured and stored at −80C. The strains were subsequently characterized by standard phenotypic methods described by Bisgaard *et al.* (1991).

On a farm rearing fattening Muscovy ducks (flock number 5) from which *P. multocida* was isolated in 1998 during this study, fowl cholera had been diagnosed in 1996 and 1997, but not in 1998. Although a multiple age operation, the farm was free of birds once per year for cleaning and disinfection. Two and six strains of *P. multocida* were isolated from Muscovy ducks that died in two distinct outbreaks of

Table 1. *Frequency of isolation and subspecies of* P. multocida *found in flocks infected with* P. multocida

Flock number	Breed of poultry and type	History	Subspecies of *P. multocida*	*P. multocida*					
				Selective medium			Mouse inoculation		
				Pharynx	Cloaca	Pharynx	Cloaca	Pharynx and cloaca	Total (%)
1	Broilers, parents	Fowl cholera	*multocida*	ND	ND	8/30	20/30	4/30	24/30 (80%)
2	Muscovy ducks, parents	Apparently healthy	*multocida*	0/8	0/8	4/8	2/8	2/8	5/8 (63%)
3	Pekin ducks, parents	Apparently healthy	*multocida*	ND	ND	14/30	9/30	4/30	19/30 (63%)
4	Geese, parents	Apparently healthy	*multocida*	ND	ND	1/30	0/30	0/30	1/30 (3%)
5	Muscovy ducks, fattening	Apparently healthy	*septica*	0/30	0/30	(16/30)[a]	(16/30)[a]	(16/30)[a]	16/30 (53%)
6	Brown layers	Fowl cholera	*septica*	0/30	1/30	5/30	21/30	4/30	22/30 (73%)
7	Broilers, parents	Apparently healthy, vaccinated against fowl cholera	*septica*	ND	ND	10/30	4/30	0/30	14/30 (47%)
8	Pekin ducks, parents	Apparently healthy	*gallicida*	ND	ND	1/30	0/30	0/30	1/30 (3%)
9	Pekin ducks, parents	Apparently healthy	*gallicida*	0/30	0/30	1/30	4/30	0/30	5/30 (17%)
10	Pekin ducks, parents	Apparently healthy	*gallicida*	ND	ND	1/30	12/30	0/30	13/30 (43%)
		Total		0/98 (0%)	1/98 (1%)	45/248 (18%)	72/278 (29%)	13/248 (5%)	120/278 (43%)

ND, Not done.

[a](16/30), Swabs from pharynx and cloaca of the same duck were mixed before inoculation, not included in the totals.

fowl cholera in 1996 and 1997, respectively, and were available for comparative investigations.

Restriction endonuclease analysis and ribotyping

Restriction endonuclease analysis and ribotyping were only performed on isolates from the aforementioned farm (Table 1, flock number 5). Chromosomal DNA analysis was performed on all 16 strains recovered from the pharynx/cloaca of a clinically normal flock of fattening Muscovy ducks in 1998, and two and six strains obtained from ducks which died of fowl cholera in 1996 and 1997, respectively. Isolation of DNA and digestion with restriction endonuclease HpaII (Boehringer-Mannheim, Mannheim, Germany) and subsequent electrophoretic separation of DNA fragments by agarose gel electrophoresis was performed as previously described by Christensen *et al.* (1993). The DNA was transferred to nylon membranes (Hybond-N, Amersham Int., Amersham, Bucks, UK) by vacuum blotting. DNA was fixed to the membrane by baking at 80C for 60 min. Ribosomal RNA (16s and 23s) from *Escherichia coli* were bought and labelled with digoxigenin using reverse transcriptase (Boehringer-Mannheim) as reported previously by Christensen *et al.* (1993). The probes were hybridized, and membrane-fixed DNA developed under the conditions described by Christensen *et al.* (1993).

Statistical analysis

Statistical analysis of data to determine significance of results was performed using the Chi-square test.

Results

The prevalence of *P. multocida* in different species of poultry and the distribution of different subspecies of *P. multocida* are summarized in Table 1. *P. multocida* was demonstrated in seven out of 20 apparently healthy flocks of web-footed birds examined (35%) and in three out of eight chicken flocks, two of which suffered from fowl cholera. Forty-five and 72 strains were obtained from pharynx and cloaca, respectively, by mouse inoculation. Sixteen more strains were obtained from a flock of Muscovy ducks from which the swabs from pharynx and cloaca of the same bird were mixed before mouse inoculation. A single strain was obtained from the cloaca by isolation from selective medium (Table 1).

P. multocida ssp. *multocida, P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida* were obtained from the cloaca and/or pharynx of four, three and three flocks, respectively (Table 1). *P. multocida* ssp. *multocida* was demonstrated in parent flocks of broilers, Muscovy ducks, Pekin ducks and geese. *P. multocida* ssp. *septica* was isolated from fattening Muscovy ducks, broiler parents and brown layers, while *P. multocida* ssp. *gallicida* was isolated from birds in three different flocks of parent Pekin ducks on the same farm. The history of the *P. multocida* positive flocks is given in Table 1.

The prevalence of *P. multocida* ssp. *multocida* carriers in the broiler parent flock (80%) affected by fowl cholera was not significantly higher ($P > 0.05$) than that of healthy flocks of Muscovy ducks (63%) and Pekin ducks (63%) (Table 1). However, the prevalence of *P. multocida* ssp. *multocida* in the goose flock was significantly lower ($P < 0.001$) than in other flocks carrying *P. multocida* ssp. *multocida*. Twenty-eight isolates were obtained from 24 broiler parents (flock number 1), where the prevalence of cloacal carriers, 20/24 (83%), was significantly higher ($P < 0.001$) than that of pharyngeal carriers, 8/24 (33%), and that of carriers in both the pharynx and cloaca, 4/24 (17%).

In apparently normal flocks of Pekin ducks, Muscovy ducks and geese (flocks number 2 to 4), 25 carriers of *P. multocida* ssp. *multocida* were found. Nineteen carriers (76%) were shown to harbour *P. multocida* in the pharynx, a significantly higher proportion ($P < 0.01$) than that of cloacal carriers, 11/25 (44%), and of carriers on the mucosa

Table 2. *Phenotypic characteristics separating different clones of* P. multocida

Flock number	1 Broiler parents	2 Muscovy ducks	3 Pekin ducks	4 Geese	5 Muscovy ducks	6 Layers	7 Broiler parents	8 White Pekin ducks	9 White Pekin ducks	10 White Pekin ducks
L(+)-Arabinose	–	–	–	–	(+)	–	–	+	+	+
D (–)-Arabinose	(+)	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)
D(+)-Xylose	+	–	+	+	(+)	+	+	–	–	–
Dulcitol	–	–	–	–	–	–	–	+	+	+
D(–)-Sorbitol	+	+	+	+	–	–	–	+	+	+
L(–)-Fucose	(+)	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)
ONPG[a]	–	+	–	–	–	–	–	–	–	–
Trehalose	+	–	–	–	+	+	+	–	–	–
PNPG[b]	+	–	–	–	+	+	+	–	–	–
Subspecies	*multocida*	*multocida*	*multocida*	*multocida*	*septica*	*septica*	*septica*	*gallicida*	*gallicida*	*gallicida*

+, all strains positive within 1 to 2 days; (+), all strains late positive (3 days); –, all strains negative within 14 days.
[a]ONPG, β-galactosidase.
[b]PNPG, α-galactosidase.

of both pharynx and cloaca, 5/25 (20%). However, the prevalence of carriers of *P. multocida* ssp. *multocida* in the cloaca was significantly higher ($P < 0.005$) than that of carriers in both the cloaca and pharynx.

Three phenotypic variants of *P. multocida* ssp. *multocida* were distinguished: one from geese and Pekin ducks; and two different variants from Muscovy ducks and a flock of broiler parents (Table 2). Differences in fermentation of D(−)-arabinose, D(+)-xylose, L(−)-fucose, trehalose, β-galactosidase (ONPG) and α-galactosidase (PNPG) separated the *P. multocida* ssp. *multocida* variants. Isolates from the same flock had identical phenotypic characteristics.

Of the three flocks with *P. multocida* ssp. *septica*, only the flock of brown layers (flock number 6) was reported to have mortalities caused by fowl cholera (Table 1). The prevalence of *P. multocida* ssp. *septica* in healthy layers in this flock (73%) was significantly higher ($P < 0.001$) than that in flock number 5, the apparently healthy fattening Muscovy ducks (53%), and flock number 7 of vaccinated broiler parents (47%). However, there was no significant difference ($P > 0.05$) between the prevalence of *P. multocida* ssp. *septica* in vaccinated broiler parents compared with fattening Muscovy ducks. Excluding results from the flock of fattening Muscovy ducks, where swabs from the pharynx and cloaca were pooled before mouse inoculation, five chickens from the flock affected with fowl cholera had *P. multocida* ssp. *septica* in the pharynx, 21 in the cloaca, and four chickens had *P. multocida* ssp. *septica* in both the pharynx and cloaca. Thus, a significantly higher number ($P < 0.001$) of chickens from the flock affected with fowl cholera carried *P. multocida* ssp. *septica* in the cloaca compared with the pharynx, while the opposite was observed for the apparently healthy, vaccinated flock of broiler parents.

Two phenotypic variants of *P. multocida* ssp. *septica* separated by late fermentation of L(+)-arabinose were identified in the present study, while strains within each flock appeared to be clonal. Fermentation of L(+)-arabinose was not observed in strains from brown layers and parent broilers, while isolates from fattening Muscovy ducks appeared to be late fermenters. The isolates from fattening Muscovy ducks were shown to be clonal by restriction endonuclease analysis and ribotyping. Comparison of the present (1998) isolates of *P. multocida* ssp. *septica* with outbreak strains from the same farm showed that a different clone of *P. multocida* ssp. *septica* was involved in one of two outbreaks that occurred in 1997. The clones differed in their reactions with L(+)-arabinose, trehalose and PNPG. *P. multocida* ssp. *multocida* was obtained from the same farm in 1996 and in the second 1997 outbreak, but these clones were different in their reactions with lactose, trehalose, ONPG, and PNPG (data not shown).

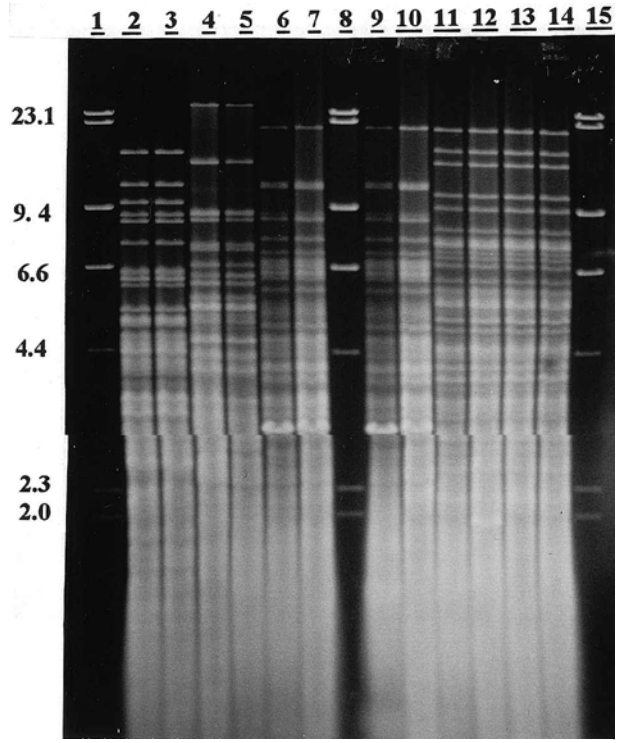

Figure 1. Hpa*II REA profiles of 1996 to 1998 strains from fattening Muscovy ducks. Lane 1, Molecular weight marker phage lambda DNA digested with* Hind*III; lanes 2 and 3*, P. multocida *ssp.* septica *from 1997 outbreak; lanes 4 and 5*, P. multocida *ssp.* multocida *from 1996 outbreak; lane 8, phage lambda DNA digested with* Hind*III*; *lanes 6 7, 9 and 10*, P. multocida *ssp.* multocida *from 1997 outbreak; lanes 11 to 14*, P. multocida *ssp.* septica *from 1998 carriers; lane 15, phage lambda DNA digested with* Hind*III*.

P. multocida ssp. *gallicida* was found in three flocks of healthy Pekin ducks originating from different houses on the same farm. Previous flocks on this farm had experienced outbreaks of fowl cholera caused by the same subspecies. The prevalence of *P. multocida* ssp. *gallicida* differed significantly ($P < 0.001$) among the three flocks. The highest prevalence was 43% in one flock, and the prevalences were 17 and 3% in the other two flocks (Table 1). Cloacal carriage was shown in 84% (16/19) of *P. multocida* ssp. *gallicida*-positive ducks, significantly higher ($P < 0.001$) than the proportion of pharyngeal carriers, 16% (3/19). No duck was found to carry *P. multocida* ssp. *gallicida* in both the pharynx and cloaca. All isolates of *P. multocida* ssp. *gallicida* obtained in this study belonged to the same phenotypic type.

Selective medium recovered only a single isolate of *P. multocida* ssp. *septica* from the cloaca of a brown layer, while isolates were obtained from 22 of 30 layers by mouse inoculation. Three other flocks (numbers 2, 5 and 9) from which isolates were obtained by mouse inoculation were negative on selective medium. The sensitivity of the selective medium was significantly lower ($P < 0.001$) than mouse passage, but only four *P. multocida*-

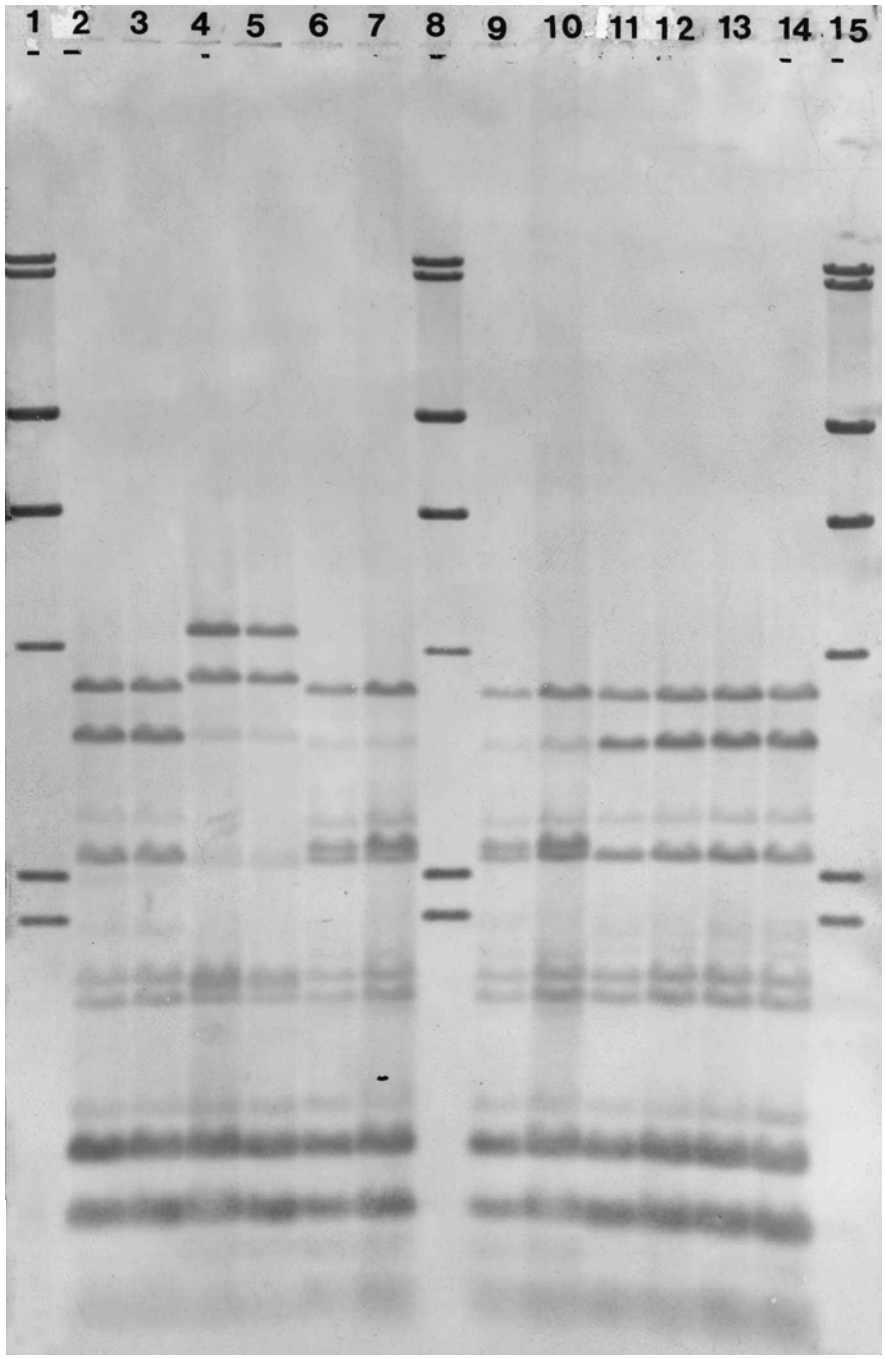

Figure 2. *The ribotypes obtained from digests in Figure 1a.* Hpa*II REA profiles of 1996 to 1998 strains from fattening Muscovy ducks. Lane 1, Molecular weight marker phage lambda DNA digested with* Hind*III; lanes 2 and 3,* P. multocida *ssp. septica from 1997 outbreak; lanes 4 and 5,* P. multocida *ssp. multocida from 1996 outbreak; lane 8, phage lambda DNA digested with* Hind*III; lanes 6 7, 9 and 10,* P. multocida *ssp. multocida from 1997 outbreak; lanes 11 to 14,* P. multocida *ssp. septica from 1998 carriers; lane 15, phage lambda DNA digested with* Hind*III.*

positive flocks were investigated by both mouse inoculation and selective medium (Table 1).

Restriction endonuclease analysis and ribotyping

Restriction endonuclease analysis (REA) and ribotyping of strains from fattening Muscovy ducks showed that the two outbreak strains of *P. multocida* ssp. *multocida* from 1996 were clonal, both by ribotyping and REA typing (Figures 1 and 2). These strains were easily differentiated from four outbreak strains from 1997 on both REA and ribotyping. Differences included several bands in both ribotyping and REA typing. These results agreed with phenotypic findings that the strains were different.

REA profiles of the outbreak clone of *P. multocida* ssp. *septica* from 1997 were also different from the 1998 clone obtained from carrier ducks, but they had the same ribotype pattern. The 1998 clone possessed the REA fragments of

approximately 23 and 16 kb that were not found in the 1997 outbreak clone. More differences were seen in the size and number of smaller fragments (Figures 1 and 2). Each of the two REA types obtained corresponded to a different phenotypic clone.

Discussion

To the knowledge of the authors, systematic investigations on the prevalence of *P. multocida* and its three subspecies have not been performed since the genus *Pasteurella sensu stricto* was reorganized by Mutters *et al.* (1985). Consequently, very little is known about the prevalence of healthy carriers. In addition, very little is known about the number of clones involved in individual outbreaks of fowl cholera. Only recently, the same clone of *P. multocida* ssp. *multocida* was shown to be involved in two outbreaks of fowl cholera in birds in Denmark (Christensen *et al.*, 1998). In the present study, all three subspecies of *P. multocida (P. multocida* ssp. *multocida, P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida*) were demonstrated in commercial poultry in Denmark. In each flock, a single phenotypic clone was found. Sixteen strains from a single flock were shown to be clonal by chromosomal DNA analysis.

In flocks affected with fowl cholera, the prevalence of *P. multocida* ssp. *multocida* in broiler parents was 80% and that of *P. multocida* ssp. *septica* in brown layers was 73%. Although these were not significantly different ($P > 0.05$) from some of the clinically normal flocks, most of the other flocks had a significantly lower prevalence ($P < 0.05$). *P. multocida* ssp. *gallicida*, which more frequently seems to be a pathogen of web-footed birds (Mutters *et al.*, 1985), was recovered only from healthy ducks, but previous flocks at the same site had experienced fowl cholera caused by the same clone (Bisgaard, unpublished data). The present results support the findings of Mraz *et al.* (1980), who found a higher prevalence of *P. multocida* in convalescent chicken flocks (25.5%), than in disease-free flocks (6.7%). From a total of 120 different birds found to carry *P. multocida*, almost equal proportions were carrying subspecies *multocida* and *septica* (49 and 52, respectively), and a smaller proportion were carrying subspecies *gallicida* (19 birds). These findings are contrary to those of Fegan *et al.* (1995), who found subspecies *multocida* to be the predominant isolate from poultry while the other two subspecies only occurred sporadically.

In flocks of broiler parents and brown layers suffering from fowl cholera caused by *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica*, respectively, the proportion of cloacal carriers were significantly greater ($P < 0.05$) than that of pharyngeal carriers. These results confirm previous observations by Bisgaard (1995) but contradict the findings by Iliev *et al.* (1964), who found that *P. multocida* was destroyed in the proventriculus and no viable form was found in the cloaca. However, further studies on colonization and multiplication of *P. multocida* are needed to clarify the pathogenesis. The present findings emphasize the importance of sampling the cloaca of birds when investigating the epidemiology of fowl cholera. Cloacal carriage may also contribute to the shedding of bacteria from the carrier, which may add to the rapid spread of infection in the flock.

The pathogenicity of *P. multocida* has been shown to be increased by bird-to-bird passage over a short period of time (Matsumoto & Strain, 1993). This may indicate that natural passage of *P. multocida* between the birds may enhance the virulence of a normally avirulent strain, leading to clinical disease in a flock of carriers. The presence of distinct clones of *P. multocida* in positive flocks in the current study indicates introduction from a common source, which was possibly followed by bird-to-bird transmission within the flocks. However, the effect of *in vivo* passage on the virulence of subspecies of *P. multocida* for different hosts remains to be investigated. Meanwhile, the presence of *P. multocida* should be viewed as a potential risk factor whenever it is found in a flock of birds.

Significant phenotypic variations have been reported previously for *P. multocida* (Heddleston, 1976; Blackall *et al.*, 1995; Christensen *et al.*, 1998). Although each flock investigated harboured a single phenotypic clone, there were overlaps where one clone affected two or more flocks. Further investigations on *P. multocida* ssp. *septica* from a flock of fattening Muscovy ducks by REA and ribotyping showed that all 1998 isolates were also clonal by DNA analysis. Comparison of phenotypic features with previous fowl cholera outbreak strains from this farm revealed two different clones of *P. multocida* ssp. *multocida* in 1996 and 1997, and a separate *P. multocida* ssp. *septica* in a different outbreak in 1997. All these strains were further shown to differ genotypically (Figure 1a). The clonal nature of *P. multocida* demonstrated in different flocks on this farm (some of which were associated with fowl cholera) suggests that multiple introductions are rare and that clones of low virulence exist. Observations from the same farm over a period of 3 years also indicated that it was possible to eliminate *P. multocida* from infected farms.

Diversity of clones of *P. multocida* involved in different fowl cholera outbreaks has been demonstrated elsewhere (Carpenter *et al.*, 1991; Blackall *et al.*, 1995). Blackall *et al.* (1995) demonstrated various subspecies, biovars and genotypes involved in fowl cholera. In that study, other common factors such as stress were considered to be responsible for precipitating outbreaks of fowl cholera, because no

correlation was found between proximity of the farms and the genotypic clones involved in the outbreaks. The presence of other factors precipitating fowl cholera might partly explain the occurrence of a clone not associated with disease subsequent to different clones associated with outbreaks on the farm of fattening Muscovy ducks (farm number 5). Furthermore, this indicates the risk of outbreaks in flocks with healthy carriers of *P. multocida*.

Induction of immunity by *P. multocida* killed vaccines has been reported to be more specific for homologous strains than for other strains (Heddleston *et al.*, 1970). In the present study, 47% of vaccinated broiler parents were shown to carry *P. multocida* ssp. *septica* on either the pharyngeal and/or cloacal mucosa. Although this prevalence is significantly lower ($P < 0.001$) than that of a flock of brown layers with fowl cholera (73%), it is uncertain whether the strains in the Pabac vaccine, which include only *P. multocida* ssp. *multocida* and *P. multocida* ssp. *gallicida* (serotypes 1,3, and 4 according to Heddleston), might have any effect on immunity to infections caused by *P. multocida* ssp. *septica*. Furthermore, the pathogenicity of the isolated strains needs to be known before any conclusions can be made on the effect of the vaccine on the development of clinical disease.

Selective media have been reported to be efficient in isolating *P. multocida* from dogs and cats (Baldrias *et al.*, 1988; Ganiere *et al.*, 1993). However, in the current study, a higher recovery rate of *P. multocida* was obtained by mouse passage. Moore *et al.* (1994) showed that double selective treatment provided by selective enrichment broth and selective agar lowers the isolation rate of *P. multocida* from both pure and contaminated samples. Inclusion of antibiotics in the medium might select for other bacteria that could inhibit *P. multocida*. In our experiments, we found that the isolates obtained by inoculation of mice, and subsequently inoculated on selective medium, grew well within 24 h of incubation. Additional studies on the interaction between *P. multocida* and the normal microflora of poultry are required to understand its effect on the selection of *P. multocida*.

In summary, this study has established the presence of *P. multocida* in poultry kept under intensive commercial management in Denmark. The birds were found to harbour *P. multocida* in either the cloaca or the pharynx and, in some birds, in both sites. Most of these isolates were obtained by mouse inoculation. Further investigations are needed to develop agar-based selective media for the isolation of *P. multocida* from avian species to avoid using experimental mice. It is recommended that when carriers of *P. multocida* are looked for, both the cloaca and the pharynx should be sampled for isolation.

References

Baldrias, L., Frost, A.J. & O'Boyle, D. (1988). The Isolation of *Pasteurella*-like organisms from the tonsillar region of dogs and cats. *Journal of Small Animal Practice, 29*, 63–68.

Bisgaard, M. (1995). Salpingitis in web-footed birds: prevalence, aetiology and significance. *Avian Pathology, 24*, 443–452.

Bisgaard, M., Abdullahi, M.Z. & Gilmour, N.J.L. (1991). Further studies on the identification of *Pasteurellaceae* from cattle lungs.*Veterinary Record 128*, 428–429.

Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N. & Morrow, C.J. (1995). Characterization of *Pasteurella multocida* isolated from fowl cholera on turkey farms. *Australian Veterinary Journal, 72*, 135–138.

Cannon, R.M. & Roe, R.T. (1982). *Livestock Disease Surveys: A Field Manual for Veterinarians.* Canberra: Australian Government Publishing Service.

Carpenter, T.E., Hirsh, D.C., Kasten, R.W., Hird, D.W., Snipes, K.P. & McCapes, R.H. (1989). *Pasteurella multocida* recovered from live turkeys: prevalence and virulence in turkeys. *Avian Diseases, 33*, 12–17.

Carpenter, T.E., Snipes, K.P., Kasten, R.W., Hird, D.W. & Hirsh, D.C. (1991). Molecular epidemiology of *Pasteurella multocida. American Journal of Veterinary Research, 52*, 1345–1349.

Christensen, J.P., Olsen, J.E. & Bisgaard, M. (1993). Ribotypes of *Salmonella enterica* serovar *gallinarum* and *pullorum. Avian Pathology, 22*, 725–738.

Christensen, J.P., Dietz, H.H. & Bisgaard, M. (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from two outbreaks of avian cholera in avifauna in Denmark. *Avian Pathology, 27*, 373–381.

Christensen, J.P., Petersen, K.D., Hansen, H.C. & Bisgaard, M. (1999). Occurrence of fowl cholera in Danish wild birds, and poultry production. Possible connections. *Dansk-Veterinaertidsskrift, 82*, 342–346

Curtis, P.E. & Ollerhead, G.E. (1981). Investigation to determine whether healthy chickens and turkeys are oral carriers of *Pasteurella multocida. Veterinary Record, 108*, 206–207.

Donahue, J.M. & Olson, L.D. (1972). Characteristics of isolates of *Pasteurella multocida* obtained on consecutive or different years from the same turkey farms. *Avian Diseases, 16*, 529–534.

Donnio, P.Y., Goff, C.L., Avril, J.L., Pouedras, P. & Gras-Rouzet, S. (1994). *Pasteurella multocida*: oropharygeal carriage and antibody response in breeders. *Veterinary Research, 25*, 8–15.

Fegan, N., Blackall, P.J. & Pahoff, J.L. (1995). Phenotypic characterization of *Pasteurella multocida* isolates from Australian poultry. *Veterinary Microbiology, 47*, 281–286.

Ganiere, J.P., Escande, F., Andre, G. & Larrat, M. (1993). Characterization of *Pasteurella* from gingival scrapings of dogs and cats. *Comparative Immunology, Microbiology and Infectious Diseases, 16*, 77–85.

Heddleston, K.L. (1976). Physiologic characteristics of 1268 cultures of *Pasteurella multocida. American Journal of Veterinary Research, 37*, 745–747.

Heddleston, K.L., Gallagher, J.E. & Rebers, P.A. (1970). Fowl cholera: immune response in turkeys. *Avian Diseases, 14*, 626–635.

Heddleston, K.L., Goodson, T., Leibovitz, L. & Angstrom, C.I. (1972). Serological and biochemical characteristics of *Pasteurella multocida* from free flying birds and poultry. *Avian Diseases, 16*, 729–734.

Hunter, B. & Wobeser, G. (1980). Pathology of experimental avian cholera in mallard ducks. *Avian Diseases, 24*, 403–414.

Iliev, T.R., Arsov, G.G. & Iovchev, E. (1964). The carrier state in fowl cholera. *Veterinary Bulletin, 34*, 129.

Matsumoto, M. & Strain, J.G. (1993). Pathogenicity of *Pasteurella multocida*; its variable nature demonstrated by in vivo passages. *Avian Diseases, 37*, 781–785.

Moore, M.K., Cicnjak-Chubbs, L. & Gates, R.J. (1994). A new selective enrichment procedure for isolating *Pasteurella multocida* from avian and environmental samples. *Avian Diseases, 38*, 317–324.

Mraz, O., Sisak, F. & Jelen, P. (1980). The *Pasteurella* carriers in farm and laboratory animals. *Comparative Immunology, Microbiology and Infectious Diseases, 2*, 437–445.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. (1985). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposal for the new species *Pasteurella dagmatis, Pasteurella stomatis, Pasteurella anatis* and *Pasteurella langaa. International Journal of Systematic Bacteriology, 35*, 309–322.

Pritchett, I.W., Beaudette, F.R. & Hughes, R.W. (1930). Epidemiology of fowl cholera. IV Field observations of the spontaneous disease. *Journal of Experimental Medicine, 52*, 249–258.

Rhoades, K.R. (1964). Microscopic lesions of acute fowl cholera in mature chickens. *Avian Diseases, 8*, 658–665.

Rimler, R.B. & Rhoades, K.R. (1989). *Pasteurella multocida.* In C. Adlam & J.M. Rutter (Eds.), *Pasteurella and Pasteurellosis*, 1st edn (pp. 37–73). London: Academic Press.

Snipes, K.P., Carpenter, T.E., Corn, L.J., Kasten, R.W., Hirsh, D.C., Hird, D.W. & McCapes, R.H. (1988). *Pasteurella multocida* in wild mammals and birds in California: prevalence and virulence for turkeys. *Avian Diseases, 32*, 9–15.

Sulong, A.K. & Maheswaran, S.K. (1976). Studies on *Pasteurella multocida.* IV. Immunofluorescence detection of the organisms in spleen and lungs of turkeys vaccinated with live oral vaccines. *Veterinary Microbiology, 1*, 3–14.

Wilson, M.A., Duncan, R.M., Nordholm, G.E. & Berlowski, B.M. (1995). Serotypes and DNA fingerprinting profiles of *Pasteurella multocida* isolated from raptors. *Avian Diseases, 39*, 94–99.

RÉSUMÉ

Investigations sur les taux de portage de *Pasteurella multocida* dans des troupeaux commerciaux de poulets sains ou affectés par le choléra

Vingt troupeaux de palmipèdes (canards Pékin, canards de barbarie et oies) ainsi que 8 troupeaux de poulets élevés de façon intensive ont été examinés pour rechercher les porteurs de *Pasteurella multocida.* Cinq cent soixante dix huit palmipèdes et 240 poulets appartenant à des troupeaux sains ou affectés par le choléra ont été analysés. Cent trente cinq souches (80 de troupeaux sains et 55 de troupeaux affectés par le choléra) ont été isolées à partir des muqueuses du pharynx et du cloaque après passage sur souris (134 souches) et par culture en milieu sélectif (1 souche). Trente cinq pour cent (7/20) des troupeaux de palmipèdes et 38 % (3/8) des troupeaux de poulets avaient des oiseaux porteurs de *P. multocida* dans le pharynx et/ou le cloaque. Les oiseaux des troupeaux affectés par le choléra étaient porteurs de *P. multocida* au niveau de la muqueuse du cloaque avec une prévalence significativement supérieure ($P < 0,001$) à celle de la muqueuse du pharynx, alors que l'opposé a été observé chez les poulets des troupeaux sains. La caractérisation phénotypique a confirmé la présence de *P. multocida* ssp. *multocida, P. multocida* ssp. *septica* et *P. multocida* ssp. *gallicida* dans les troupeaux soumis à analyse. *P. multocida* ssp. *gallicida* a été exclusivement isolée de canards Pékin alors que *P. multocida* ssp. *multocida* et *P. multocida* ssp. *septica* ont été isolées aussi bien chez des poulets que chez des palmipèdes. Il a été observé que chaque troupeau a été infecté par un seul clone phénotypique, mais quelques clones ont été isolés à partir de plus d'un troupeau. Un clone différent a été trouvé à partir de 4 bandes atteintes de choléra dans une des fermes les deux années précédentes. Deux clones génotypiques et phénotypiques appartenant chacun à *P. multocida* ssp. *multocida* et *P. multocida* ssp. *septica* ont été trouvés. Cette observation indique que les cas sont généralement dus à un clone et que l'élimination de *P. multocida* des fermes infectées est possible. Les résultats suggèrent que des volailles saines au contact de porteurs convalescents, puissent également devenir des porteurs de *P. multocida.* Cependant, la virulence des isolats de *P. multocida* et la résistance des porteurs à l'infection clinique nécessitent d'être examinées. Ceci est le premier rapport de l'isolement de *P. multocida* à partir d'une muqueuse cloacale de volaille domestique apparemment saine. Les échantillons de cloaque sont apparus être sensibles pour la détection des porteurs de *P. multocida.* Bien que des milieux sélectifs aient été utilisés à une petite échelle, les résultats suggèrent que l'inoculation à la souris ait été une méthode plus efficace que l'utilisation de milieux sélectifs pour l'isolement de *P. multocida* à partir de volailles.

ZUSAMMENFASSUNG

Untersuchungen über die Rate von Pasteurella multocida-Trägern in gesunden Wirtschaftsgeflügelherden und an Hühnercholera erkrankten Herden

Zwanzig Herden schwimmfüßiger Vögel (Peking- und Moschusenten und Gänse) und 8 Hühnerherden, unter intensiver Betreuung aufgezogen, wurden auf das Vorhandensein von Pasteurella multocida-Trägern untersucht. Fünfhundertachtundsiebzig schwimmfüßige Vögel und 240 Hühner sowohl aus gesunden Herden als auch aus Herden mit Geflügelcholera wurden untersucht. Insgesamt 135 Isolate (80 aus gesunden Herden und 55 aus Herden mit Geflügelcholera) wurden von der Rachen- und Kloakenschleimhaut nach Mäusepassagen (134 Isolate) und nach der Kultivierung auf Selektivnährböden (ein Isolat) gewonnen. In 35% (7/20) der Herden von schwimmfüßigen Vögeln und 38% (3/8) der Hühnerherden gab es Tiere, die P. multocida im Rachen und/oder in der Kloake hatten. Vögel aus Herden mit Geflügelcholera trugen P. multocida mit signifikant höherer Prävalenz in der Mukosa der Kloake ($P < 0,001$) als im Rachen, während das Gegenteil bei den Tieren aus gesunden Herden festgestellt wurde. Ausgedehnte Phänotyp-Charakterisierungen bestätigten das Vorhandensein von P. multocida ssp. multocida, P. multocida ssp. septica und P. multocida ssp. gallicida in den untersuchten Herden. P. multocida ssp. gallicida wurde ausschließlich von Pekingenten isoliert, während P. multocida ssp. multocida und P. multocida ssp. septica sowohl von Hühnern als auch von schwimmfüßigen Vögeln gewonnen wurden. Es wurde nachgewiesen, dass jede Herde mit einem einzigen Phänotyp-Klon infiziert war, aber manche Klone wurden in mehr als einer Herde gefunden. Bei jedem von vier Geflügelcholera-Ausbrüchen in den vorhergehenden Jahren wurde ein anderer Klon festgestellt. Je zwei genotypische und phänotypische Klone von P. multocida ssp. multocida und P. multocida ssp. septica wurden nachgewiesen. Diese Beobachtung zeigte, dass die Ausbrüche gewöhnlich klonal sind, und dass die Eliminierung von P. multocida aus infizierten Farmen möglich ist. Die Ergebnisse lassen darauf schließen, dass neben den Rekonvaleszenzausscheidern auch gesundes Geflügel Keimträger sein kann. Die Virulenz von P. multocida-Isolaten und die Resistenz der Ausscheider gegen die klinische Infektion muss noch untersucht werden. Dies ist der erste Bericht über die Isolierung von P. multocida aus der Kloakenschleimhaut von anscheinend gesundem Hausgeflügel. Kloakenproben schienen für die Ermittlung von P. multocida-Ausscheidern empfindlicher zu sein. Obgleich Selektivnährböden nur in begrenztem Umfang verwendet wurden, lassen die Ergebnisse darauf schließen, dass der Mäuseinfektionsversuch eine leistungsfähigere Methode für die Isolierung von P. multocida aus Geflügel ist als die Verwendung von Selektivnährböden.

RESUMEN

Estudio del indice de portadores de *Pasteurella multocida* en manadas sanas de pollos comerciales y en manadas afectadas por colera aviar

Se examinaron veinte manadas de palmípedas (patos de Pekín, patos almizclados y ocas) y 8 manadas de pollos en régimen de explotación intensiva para determinar la presencia de *Pasteurella multocida.* Se evaluaron quinientas setenta y ocho palmípedas y 240 pollos procedentes de manadas sanas así como de las manadas afectadas por el cólera aviar. Se obtuvo un total de 135 aislados (80 de manadas sanas y 55 de manadas afectadas) a partir de mucosa faríngea y cloacal, después de un pase en ratón (134 aislados) y cultivo en medio selectivo (un aislado). Un treinta y cinco por ciento (7/20) de las manadas de palmípedas y un 38% (3/8) de las manadas de pollos presentaban animales portadores de *Pasteurella multocida* en faríngе y/o cloaca. Las aves afectadas por cólera aviar presentaban *P. multocida* con mucho mayor prevalencia en la mucosa de la cloaca ($p < 0.001$) que en la mucosa faríngea, mientras que ocurría lo contrario en las manadas sanas. Una mayor caracterización fenotípica confirmó la presencia de *P. multocida ssp. multocida, P. multocida ssp. septica* y *P. multocida ssp. gallicida* en las manadas evaluadas. La *P. multocida ssp. gallicida* se aisló exclusivamente en patos de Pekin, mientras que la *P. multocida ssp. multocida* y la *P. multocida ssp. septica* se aisló en tanto en pollos como en palmípedas. Cada manada estaba infectada por un solo clon

fenotípico, aunque algunos clones se encontraron en más de una manada. En una de las granjas se detectó un clon diferente en cada uno de los cuatro brotes de cólera aviar habidos en los dos años precedentes. Se identificaron dos clones fenotípicos y genotípicos de *P. multocida ssp. multocida* y *P. multocida ssp. septica*. Esta observación indicaba que los brotes son normalmente clonales y que la eliminación de *P. multocida* de las granjas infectadas es posible. Los resultados son sugestivos de que las aves sanas, además de las convalecientes portadoras, pueden ser portadoras de *P. multocida*, aunque la virulencia de los diferentes aislados de *P. multocida* y la resistencia de los portadores a la infección clínica queda por ser estudiada. Este es el primer estudio acerca de el aislamiento de *P. multocida* a partir de mucosa cloacal en pollos aparentemente sanos. Aparentemente la toma de muestras a partir de cloaca presenta una mayor sensibilidad para la detección de portadores de *P. multocida.* Aunque la utilización de un medio selectivo se aplicó sólo en algunos casos, los resultados son sugestivos de que la inoculación en ratón fue mucho más eficiente para *P. multocida* en pollos que el uso de medios selectivos.