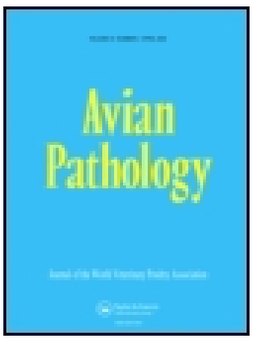

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Serum resistance of *Pasteurella multocida* in avian and porcine sera, and comparative virulence investigations of selected serum-sensitive and resistant strains in chickens

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Growth in serum of *Pasteurella multocida* and related species in chicken, turkey, duck and pig sera were compared, and selected serum-resistant and serum-sensitive strains were inoculated into 18-week-old layers. Eighty-seven field strains of *Pasteurella* spp. and nine reference strains representing different clones defined by restriction endonuclease analysis (REA) profiles were used in the study. Serum activity was measured by changes in the optical density (OD) of the serum after inoculation and incubation at 41°C for chicken, turkey and duck serum and 39°C for pig serum. Serum activity was measured by comparison with previously determined serum-resistant (P-1059) and serum-sensitive (CU vaccine) strains, and classified into highly serum-resistant, moderately serum-resistant and serum-sensitive. Strains of the same REA type were found to have identical growth curves and the same maximum OD values when tested in serum from the same host species. Turkey serum was shown to be less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera. Serum-resistant strains were demonstrated among avian as well as mammalian strains. Among the avian strains, the proportion of serum-resistant strains was higher in outbreak strains than in strains from apparently healthy carriers. Removal of the capsule from selected strains by hyaluronidase treatment failed to change the serum activity. The most severe lesions in experimentally infected chickens were produced by a serum-resistant strain; however, lesions were also found in chickens infected by serum-sensitive strains, indicating the involvement of multiple factors in the virulence of *P. multocida*. Further investigations on serum resistance are indicated in order to relate other host and bacterial factors responsible for the development of fowl cholera.

Introduction

Attempts to understand the determinants of virulence factors of *Pasteurella multocida* obtained from fowl cholera have met with limited success. The role of the capsule as a virulence-contributing factor in fowl cholera has been recognized for a long time; however, other factors such as serum resistance, which also seem to be important in virulence (Hansen & Hirsh, 1989; Morishita *et al.*, 1990), have not received comparable attention. Correlation between serum resistance and virulence in animals has been demonstrated for some strains of *P. multocida* (Lee *et al.*, 1988a; Diallo & Frost, 2000) and other Gram-negative bacteria, for example *Escherichia coli* (Ellis *et al.*, 1988) and *Yersinia ruckeri* (Davies, 1991). Lee *et al.* (1988a) demonstrated that highly virulent strains of *P. multocida* obtained from turkey were resistant to turkey serum, reaching a higher optical density (OD) value than the avirulent strains. Subsequent investigations by Morishita *et al.* (1990) showed the presence of

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serum-resistant virulent *P. multocida* strains among isolates obtained from wild animals in turkey premises. In the same study, less virulent strains were found to have a lower survival rate in turkey serum. Serum-resistant strains are thus assumed to have a survival advantage in the blood system of the host, which allows them to proliferate and produce disease (Taylor, 1983).

Severity and incidence of *P. multocida* infection is known to vary considerably among different species of birds (Matsumoto *et al.*, 1991; Rimler & Glisson, 1997; Petersen *et al.*, 2001). Turkeys are considered to be the most susceptible, together with ducks, while chickens seem to be the least susceptible of the three. Comparison of the growth of similar strains in sera from different avian hosts may provide more insights into the susceptibility of different avian species to *P. multocida* infection. Knowledge of the behaviour in avian sera of strains from hosts supposed to be of importance in the epidemiology of fowl cholera may also be of help in the identification of hosts of real importance.

The present study aimed to compare serum resistance of diverse *P. multocida* strains and related species in the sera from chickens, turkeys, ducks and pigs. The results were subsequently correlated with genotype and source of the strain. Correlation between serum activity, virulence, and persistence in trachea and cloaca was investigated in experimentally infected chickens.

Materials and Methods

Serum resistance assays

Bacteria. A total of 87 field strains of *Pasteurella* spp. and nine reference strains (Table 1) were investigated for growth in sera obtained from chickens, ducks, turkeys and pigs. Field strains included isolates from free-ranging chickens and ducks in Tanzania, and dogs and cats kept in contact with them (Muhairwa *et al.*, 2001a), and outbreak and carrier strains obtained from commercial poultry flocks in Denmark (Muhairwa *et al.*, 2000). After initial isolation from the hosts, the strains were stored at –80°C and were subcultured once for use in serum resistance experiment. The selected field strains represented clones defined by restriction endonuclease analysis (REA) patterns and ribotypes as described previously (Muhairwa *et al.*, 2001b). Reference strains included serum-sensitive strains, *E. coli* (K12) (Diallo & Frost, 2000), *P. multocida* (CU vaccine strain) and a serum-resistant strain of *P. multocida* (P-1059) (Hansen & Hirsh, 1989). Other reference strains included an outbreak clone (P-40605) from wild birds in Denmark (Christensen *et al.*, 1998), and the type strains of *P. multocida* ssp. *multocida* (NCTC 10322^T), *P. multocida* ssp. *septica* (NCTC 11995^T), *P. multocida* ssp. *gallicida* (HIM 830–7^T), *Pasteurella gallinarum* (ATCC 13361^T), *Pasteurella canis* (NCTC 11621^T) and *Pasteurella stomatis* (HIM 657^T).

Collection of serum. Apparently healthy broilers (5 weeks old), ducks (7 weeks old) and turkeys (20 weeks old) from farms with no previous history of *P. multocida* infection were used for blood collection. The collected blood was allowed to clot at room temperature for 1 h, then cooled to 4°C for 1 h followed by centrifugation and filter sterilization (0.2 μl). Pooled pig serum was obtained from *P. multocida*-free pigs kept at the Danish Veterinary Laboratory, Copenhagen, Denmark. All sera were stored at –20°C, and each time before use they were thawed and filter sterilized.

Measurement of serum activity of Pasteurella strains. Preliminary assays were conducted using serum-sensitive and serum-resistant reference strains to determine the concentration of bacteria required for inoculation into the sera. The growth of these strains in serum from individual birds was also examined. Strains of the same REA type and recovered from animals of the same species were compared to determine the correlation between serum activity and the genotype.

Lytic action of complement in chicken, duck, turkey and pig sera was monitored by inoculation of 10 μl test organisms containing approximately 10^6 colony forming units (CFU)/ml into 200 μl normal serum, heat-inactivated serum (56°C for 30 min), and brain-heart infusion (BHI) broth in a microtitre plate. Duplicate suspensions of each strain were subsequently placed in a Bioscreen microplate turbidometer (Labsystems, Finland), and incubated overnight at 41°C for chicken, duck and turkey serum, and at 39°C for porcine serum. BHI broth growth controls were performed at 41°C for avian sera and at 39°C for porcine serum. Bacterial lysis and growth were monitored by changes in turbidity every 10 min and recorded automatically in BioLink (Labsystems Computer software). The data were subsequently transferred into the Excel program (Microsoft Corporation, 1999) and growth curves were analysed. The experiment was repeated twice on separate days to test the reproducibility of the results.

The strains were classified into serum-resistant and serum-sensitive by comparison with the reference serum-resistant strain *P. multocida* (P-1059) and serum-sensitive CU vaccine strain (Hansen & Hirsh, 1989). Highly serum-resistant strains included strains that had OD values equal to or above that of strain P-1059, and moderate serum-resistant strains had OD values below that of P-1059 but above that of CU strain. The strains with ODs equal to or below that of the CU strain were considered to be serum-sensitive (Table 2).

Effect of the capsule on serum resistance. Five serum-sensitive strains and five serum-resistant strains were treated with hyaluronidase to study the effect of the capsule on serum resistance. Two previously serotyped strains P-1059 (A:3) and P 40506 (A:3) were among the serum-resistant strains used as reference for capsulated strains. The capsular material was removed from encapsulated strains by growth in BHI broth containing 100 U/ml hyaluronidase (Sigma Chemical Co.) (Poermadjaja & Frost, 2000). The presence of a capsule in the normal and digested bacteria was demonstrated by staining with 1% crystal violet (Sigma) as described by Jasmin (1945). After hyaluronidase treatment, the serum activities of the strains were determined as already described.

Experimental infection of chickens

Chickens. Fifty 18-week-old layers from a *P. multocida*-free flock were used for experimental infection. Prior to the experiment, the trachea and cloacae of all chickens were swabbed, and examination for the presence of *P. multocida* was examined by mouse passage as described by Muhairwa *et al.* (2000). Briefly, the swabs were transferred into BHI and vortexed before 0.25 ml was injected intraperitoneally into Balb Cj mice raised at the department. Chickens were divided into five groups of 10 chickens, four groups were inoculated with different strains of *P. multocida* (Table 3), while the control group was inoculated with sterile BHI broth.

Selection of strains. Four *P. multocida* ssp. *multocida* strains were selected for experimental infection of chickens (Table 3). These included two serum-resistant strains, one obtained from a cat (MC 6BA) while the other represented a clone (P-40605) obtained from fowl cholera in wild birds (Christensen *et al.*, 1998), and two serum-sensitive strains obtained from a duck (Mamo 2) and a cat (KC 14Hpg). All stock strains had been stored at –80°C since initial isolation.

Preparation of cultures for inoculation. Before each experiment, each strain was thawed and plated on 5% calf blood agar (Tryptose blood agar Base; Difco Laboratories, Michigan, USA) and incubated aerobically at 37°C overnight to check for purity. Three to five colonies were then inoculated into 10 ml BHI broth and incubated overnight at 37°C with moderate shaking. The overnight broth was diluted 1 : 50 in BHI broth preheated to 41°C and then incubated aerobically at 37°C with moderate shaking to the mid-exponential phase. When OD_{410}

Table 1. *Serum activity observed for different* Pasteurella *species in the serum from chickens, ducks, turkeys and pigs*

Species (number of strains)	Source host (number of strains)	Chicken serum			Duck serum			Turkey serum			Pig serum		
		R[a]	M[b]	S[c]	R	M	S	R	M	S	R	M	S
P. multocida ssp. *multocida* (50)	Chickens (3)	–	–	3	–	–	3	–	1	2	–	–	3
	Ducks, Tanzania (3)	–	–	3	–	–	3	–	–	3	–	–	3
	Ducks, Denmark (3)	2	–	1	2	–	1	2	1	–	2	–	1
	Outbreak P-40605	1	–	–	1	–	–	1	–	–	–	–	1
	Cats (37)	3	–	34	3	2	32	3	31	3	2	3	32
	Dogs (2)	–	–	2	–	–	2	–	1	1	–	–	2
	NCTC 10322^T	–	–	1	–	–	1	–	1	–	–	–	1
P. multocida ssp. *septica* (29)	Ducks (3)	–	–	3	–	2	1	–	2	1	–	1	2
	Dogs (2)	–	–	2	–	–	2	–	2	–	–	–	2
	Cats (23)	4	–	19	3	2	18	4	16	3	2	2	19
	NCTC 11995^T	–	–	1	–	–	1	–	1	–	–	1	–
P. multocida ssp. *gallicida* (1)	HIM 830–7^T	–	–	1	–	–	1	–	1	–	–	–	1
	Subtotal *P. multocida*	10	–	70	9	6	65	10	57	13	6	7	67
P. gallinarum (2)	ATCC 13361^T	–	–	1	–	1	–	–	1	–	–	1	–
	Ducks, Tanzania (1)	–	1	–	–	–	1	–	–	1	–	–	1
P. canis (5)	Dogs (4)	–	2	2	–	–	4	–	1	3	–	–	4
	NCTC 11621^T	–	1	–	–	–	1	–	–	1	–	–	1
P. stomatis (2)	Dogs, Tanzania (1)	–	1	–	–	1	–	–	1	–	–	–	1
	HIM 657^T	–	–	1	1	–	–	–	1	–	–	–	1
P. dagmatis (4)	Dogs (4)	3	–	1	1	3	–	4	–	–	3	–	1
Total		13	5	75	11	11	71	14	61	18	9	8	76

[a] R, serum-resistant strains; [b] M, moderately serum-resistant strains; [c]S, serum-sensitive strains. Strains P-1059, CU vaccine, and *E. coli* K12 are not shown in the table.

corresponding to 2×10^8 was reached, 1 ml was diluted serially in 9 ml broth to a final concentration of 2×10^4 CFU/ml. An estimate of the bacterial count in the inoculum was calculated from the average of duplicate dilutions of each strain. The chickens in each group were separately inoculated intratracheally with 0.5 ml containing approximately 10^4 CFU respective strain. Control birds were inoculated intratracheally with 0.5 ml sterile BHI.

Parameters measured. Twenty-four hours after inoculation, five randomly selected chickens in each group were killed by decapitation and a postmortem examination performed to assess the development of the lesions. The remaining birds were screened for *P. multocida* in the trachea and cloaca at 24 h, 7 days and 14 days after infection (Table 3). Detection of *P. multocida* in the trachea and cloaca was by mouse inoculation as already described. At the end of the experiment, the remaining birds were also killed by decapitation and subjected to postmortem examination.

Statistical analysis

The chi-square test was used to compare the proportions of serum-resistant and serum-sensitive *P. multocida* strains in chicken, duck, turkey and pig sera.

Results

Serum resistance study

Preliminary serum activity studies. No variation in the growth pattern and maximum OD values was observed when the same strain was inoculated in sera from different birds of the same species. Consequently, sera of each species were pooled together for use in the major study. Strains of identical REA types were found to have identical growth curves and the same maximum OD values when cultured in serum from the same host species. Different concentrations of inocula attempted before the major experiment showed that a final concentration of about 10^4 CFU/ml in the sera and BHI broth resulted in a smooth growth curve. Very low doses resulted in a prolonged lag phase.

Growth of the Pasteurella strains in sera from different animals. In BHI broth and heat-treated sera from chickens, ducks, turkeys and pigs, all *Pasteurella* spp. strains were able to grow as indicated by changes in the turbidity of samples. No *Pasteurella* strain was completely killed in unheated chicken and pig serum. However, one *P. multocida* ssp. *multocida* strain and one *P. canis* strain were killed in duck serum and two *P. canis* strains were killed in turkey serum. Maximum OD values of investigated strains ranged from 0.5 to 0.7 in BHI broth, from 0.3 to 2.0 in chicken serum, from 0.2 to 2.0 in duck serum, from 0.1 to 2.0 in

Table 2. *Maximum OD[a] values of representative* Pasteurella *strains showing serum-resistant, moderately serum-resistant, and serum sensitive strains in chicken, duck, turkey and porcine sera. Comparative OD values of the strains in BHI[b] broth are also shown.*

					BHI	Chicken		Duck		Turkey		Pig[d]	
No	Strain	Species	Host	Reference	OD	OD	SA[c]	OD	SA	OD	SA	OD	SA
1	P-1059	*P. multocida* ssp. multocida	Turkey	Hansen &Hirsh (1989)	0.7	1.3	R	1.3	R	1.6	R	0.65	R
2	KC 22 Hpg	*P. multocida* ssp. multocida	Cat	Muhairwa *et al.* (2001b)	0.7	1.8	R	1.2	R	2	R	0.6	M
3	74782–1	*P. multocida* ssp. multocida	Duck	Muhairwa *et al.* (2000)	0.67	1.8	R	2.1	R	1.75	R	0.65	R
4	MMC 2	*P. multocida* ssp. multocida	Cat	Muhairwa *et al.* (2001b)	0.5	1.7	R	1.4	R	2	R	0.7	R
5	71840–1	*P. multocida* ssp. multocida	Duck	Muhairwa *et al.* (2000)	0.7	1.7	R	2.1	R	1.75	R	0.65	R
6	P-40605–1	*P. multocida* ssp. multocida	Eider duck	Christensen *et al.* (1998)	0.65	1.5	R	1.3	R	1.9	R	0.4	S
7	MC 6BA	*P. multocida* ssp. multocida	Cat	Muhairwa *et al.* (2001b)	0.6	1.8	R	2	R	1.9	R	0.7	R
8	KC 23aBA	*P. multocida* ssp. septica	Cat	Muhairwa *et al.* (2001b)	0.5	1.6	R	2	R	1.9	R	0.75	R
9	KC 19 Hpg	*P. multocida* ssp. septica	Cat	Muhairwa *et al.* (2001b)	0.6	1.8	R	1.2	M	1.9	R	0.4	S
10	MMC 3	*P. multocida* ssp. septica	Cat	Muhairwa *et al.* (2001b)	0.4	1.8	R	2.1	R	1.9	R	0.85	R
11	MC 2 Hpg	*P. multocida* ssp. septica	Cat	Muhairwa *et al.* (2001b)	0.6	1.7	R	1.2	R	2	R	0.6	S
12	MMD 21	*P. dagmatis*	Dog	Muhairwa *et al.* (2001b)	0.7	2	R	1.2	M	1.9	R	0.85	R
13	KD 21cBA	*P. dagmatis*	Dog	Muhairwa *et al.* (2001b)	1	1.9	R	1.3	R	1.9	R	1.2	R
14	ND 11bBA	*P. dagmatis*	Dog	Muhairwa *et al.* (2001b)	0.5	1.8	R	1.2	M	1.9	R	0.2	M
15	Mao 26	*P. gallinarum*	Duck	Muhairwa *et al.* (2001b)	0.6	0.9	M	0.4	S	0.1	S	0.3	M
16	MD 20c BA	*P. canis*	Dog	Muhairwa *et al.* (2001b)	0.7	1	M	0.4	S	–	S	0.3	S
17	KMD 25	*P. stomatis*	Dog	Muhairwa *et al.* (2001b)	0.4	0.9	M	1.2	M	1	M	0.4	S
18	CU strain	*P. multocida*	Turkey	Hansen & Hirsh, (1989)	0.5	0.75	S	0.55	S	0.65	S	0.45	S
19	MBO 3	*P. multocida* ssp. multocida	Chicken	Muhairwa *et al.* (2001b)	0.6	0.7	S	0.3	S	1.1	M	0.35	S
20	71660 3a	*P. multocida* ssp. multocida	Duck DK	Muhairwa *et al.* (2000)	0.5	0.55	S	0.4	S	1.1	M	0.4	M
21	MD 11c BA	*P. multocida* ssp. multocida	Dog	Muhairwa *et al.* (2001b)	0.6	0.5	S	–	S	0.4	S	0.3	S
22	NC 4BA	*P. multocida* ssp. multocida	Cat	Muhairwa *et al.* (2001b)	0.5	0.45	S	0.3	S	0.3	S	0.3	S
23	Mbmo 42	*P. multocida* ssp. multocida	Chicken	Muhairwa *et al.* (2001b)	0.7	0.35	S	0.4	S	0.6	S	0.4	S
24	Mamo 2	*P. multocida* ssp. multocida	Duck	Muhairwa *et al.* (2001b)	0.7	0.35	S	0.4	S	1.1	M	0.4	S
25	MBO 39 Hpg	*P. multocida* ssp. multocida	Chicken	Muhairwa *et al.* (2001b)	0.7	0.3	S	0.2	S	0.6	S	0.4	S
26	77263	*P. multocida* ssp. septica	Duck DK	Muhairwa *et al.* (2000)	0.7	0.6	S	0.4	S	0.5	S	0.5	M
27	BM 3	*P. multocida* ssp. septica	Duck DK	Muhairwa *et al.* (2000)	0.6	0.55	S	1.1	M	1.1	M	0.4	S
28	ND 15b	*P. canis*	Dog	Muhairwa *et al.* (2001b)	0.6	0.7	S	–	S	1.1	M	0.35	S
29	ND 13bBA	*P. dagmatis*	Dog	Muhairwa *et al.* (2001b)	0.5	0.2	S	2.4	R	2	R	0.9	R
30	K12	*E. coli*			0.8	0.5[b]	S	–	S	–	S	–	S

[c] SA, serum activity: R, serum resistant; M, moderately serum resistant; S, serum sensitive. –, Bacteria were completely killed.
[d] Pig sera and their BHI controls were incubated at 41°C.
[e] A short phase of growth was followed by complete killing.

turkey serum, and from 0.2 to 1.2 in pig serum (Table 2). Heat-inactivated sera resulted in a shorter lag phase and slightly higher maximum OD values than those of unheated sera. The serum-sensitive reference strain *E. coli* K12 demonstrated a short phase of growth followed by inhibition of growth in chicken serum and was completely inhibited in sera from other animals. However, this strain reproduced in all heat-inactivated tested sera and BHI broth, indicating the presence of complement bactericidal effect in the non-inactivated sera tested. The serum-sensitive reference strain of *P. multocida*, the CU vaccine strain, grew in all test sera, but the maximum OD values obtained were lower than those of the reference serum-resistant strain P-1059 (Table 2).

Significance of the capsule. Digestion of the capsule by treatment with hyaluronidase was demonstrated from strains P-1059 and P-40605. Typical capsules were not demonstrated in any of the remaining eight strains either before or after hyaluronidase treatment; however, there was no change in the maximum OD values of all 10 strains tested.

Statistical analysis

Sixty-seven out of 80 *P. multocida* strains were found to be serum resistant (highly or moderately resistant) in turkey serum (Table 1), which was statistically significantly higher ($P < 0.001$) than the proportion of the serum-sensitive strains when the same strains were grown in sera from chicken (10/80), duck (15/80) and pig (13/80). Considered together, the remaining *Pasteurella* species showed no statistically significant ($P > 0.05$) difference in the proportion of serum-resistant to serum-sensitive strains in chicken, turkey and duck sera (Table 1). However, these strains were more sensitive to pig serum ($P < 0.05$).

Experimental infection results

Pathological findings. In the group inoculated with the serum-resistant strain (MC 6BA), one chicken was found dead after 24 h, while no mortality was observed in the remaining groups (Table 3). Variations in the severity of pathological lesions were evident among the chickens infected with different strains both after 24 h and after 2 weeks.

Lesions observed in chickens inoculated with serum-resistant strains. Twenty-four hours after infection, the chickens infected with serum-resistant strains had congestion in the trachea, and three out of five chickens infected with strain P-40506 had haemorrhagic tracheitis. The feline isolate, MC 6BA, caused unilateral fibrinous pleuritis and airsacculitis in all chickens. Two chickens had hepatomegaly with diffuse multifocal greyish foci on the liver surface. Enlarged spleens with multifocal grey lesions were also observed. *P. multocida* was re-isolated from the spleens of two chickens although not from the chicken that was found dead. Strain P-40605 caused more severe lung lesions, characterized by unilateral fibrinous pleuropneumonia. The livers of four chickens were pale, enlarged and friable, and *P. multocida* was re-isolated from the spleens of all five. Multifocal grey lesions were observed on the spleen of one chicken.

In chickens killed after 2 weeks, those inoculated with strain MC 6BA strain had unilateral mild pleuritis, which was localized on the caudal margin of the lung lobes. One bird among the chickens infected with strain P-40605 was on sternal recumbency. All these chickens had unilateral lesions in the lungs, which included oedema and fibrinous pleuritis. The recumbent chicken had unilateral diffuse lung necrosis, and *P. multocida* was re-isolated from its spleen.

Lesions in chickens infected with serum-sensitive strains. In chickens killed after 24 h, strain KC 14Hpg caused fibrinopurulent pleuritis and airsacculitis, which were unilateral in two chickens and bilateral in the remaining three chickens. Comparatively, the lesions were more severe than those of strain MC 6BA but less severe than those of strain P-40605. Liver and spleen enlargement were conspicuous in one chicken, which also had multifocal grey lesions on the spleen. *P. multocida* was re-isolated from the spleen of two of the five chickens infected with strain KC 14Hpg. Strain Mamo 2 caused the least severe lesions, which included marginal pleuritis in the caudal lobes of the lungs in all infected birds. *P. multocida* was isolated from the spleen of one chicken.

Chickens killed after 2 weeks in groups infected with strain KC 14Hpg had fibrinous pleuritis in all birds and unilateral diffuse necrosis of the lungs in two chickens. Slight oedema was observed in the lungs of chickens infected with strain Mamo 2. *P. multocida* was not re-isolated from the spleen in either group.

Carriers of different strains following infection. With the exception of the group infected with strain MC 6BA, *P. multocida* was re-isolated from at least one chicken in the other groups (Table 3). However, only strain P-40605 persisted in the trachea of chickens until the end of the experiment. Only one bird that was in sternal recumbency for 2 weeks after infection had *P. multocida* in both the trachea and cloaca. All others samples were negative for *P. multocida* from the cloaca.

Discussion

Studies of complement activity to *P. multocida* have been carried out by inoculation of a suspension of viable organisms into serum and by determination of

Table 3. *Mortality and carrier status of chickens experimentally infected with different strains of* P. multocida *ssp.* multocida

Source	Strain	Mortality	Serum activity	Isolation of *P. multocida* from the spleen		Trachea carriers[a]		
				24 h	14 days	24 h	7 days	14 days
Cat	MC 6BA	1/5	R	2/5	0/5	0/5	0/5	0/5
Eider duck	P-40605–1	0/5	R	5/5	1/5	4/5	5/5	1/5[b]
Cat	KC 14Hpg	0/5	S	2/5	0/5	3/5	0/5	0/5
Duck	Mamo2	0/5	S	1/5	0/5	1/5	0/5	0/5
	Control BHI	0/5	–	0/5	0/5	0/5	0/5	0/5

R, serum resistant; S, serum sensitive.
[a] All chickens sampled negative from the cloaca, with one exception (see [b]).
[b] *P. multocida* isolated also from the cloaca.

surviving organisms, either by counting viable cells (Morishita *et al.*, 1990; Diallo & Frost, 2000) or by measuring changes in the optical density (Lee *et al.*, 1988a,b). Classification of strains into serum-sensitive and serum-resistant seems to be arbitrary, because of variations in detection techniques, inoculation dose and incubation time used by different workers (Lee *et al.*, 1988b; Morishita *et al.*, 1990; Diallo & Frost, 2000). Lack of a standardized technique for estimating serum activity of *P. multocida* makes meaningful comparison between studies difficult. However, the findings obtained from both viable cell counts and OD changes have been successfully correlated with virulence in poultry (Lee *et al.*, 1988a,b; Morishita *et al.*, 1990). Neither method indicates the actual amount of complement deposited on the bacterium. How this affects the classification of strains into serum-sensitive and serum-resistant is not known, but failure to correlate serum activity with virulence has been reported (Morishita *et al.*, 1990; Diallo & Frost, 2000). Direct detection of deposited complement components C6 and C9 on the bacterial surface by immunofluorescence (Kraiczy *et al.* 2000) was demonstrated to be useful in determining the activity of human serum on *Borrelia burgdorferi*. Development of similar complement detection techniques may help to improve studies of *P. multocida* serum resistance in different animal species.

This study has demonstrated for the first time that turkey serum was less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera (Table 1). Sixty-seven out of 80 strains of *P. multocida* (including all three subspecies) had higher OD values than the avirulent serum-sensitive CU vaccine strain (Hansen & Hirsh, 1989) in the turkey serum (Table 1). In chicken, duck and pig sera, only 10, 15 and 13 *P. multocida* strains, respectively, had OD values higher than the CU vaccine strain. Earlier studies showed that chicken serum was not inhibitory to *P. multocida* while cattle, horse, swine and rabbit sera had various degrees of inhibition (Ryu, 1959). Findings based on a single strain by Diallo & Frost (2000) showed that a *P. multocida* strain sensitive to chicken serum was resistant in turkey, sheep, bovine and rabbit serum. Different assay techniques and the limited number of strains employed in those studies do not allow safe comparison with the present results. However, the results indicate that sera from different animals vary in their reactions to *P. multocida*, and it can be concluded that the higher susceptibility of turkeys to fowl cholera correlates with their lower serum activity against *P. multocida* strains. As *P. multocida* has been demonstrated to bind iron-chelating proteins such as transferrin (Ogunnariwo *et al.*, 1991), differences in the amounts of iron acquired from different species sera should be investigated to understand the role iron acquisition in growth of *P. multocida* in animal sera.

The present study has shown that three out of five (60%) *P. multocida* strains obtained from fowl cholera outbreaks were serum resistant in all bird sera, while all nine avian carrier strains were serum sensitive. Separate studies with outbreak strains found that 60% of the strains were resistant to turkey serum (Lee *et al.*, 1988a) and 88% resistant to chicken serum (Diallo & Frost, 2000). It can be postulated that serum-resistant strains are more prevalent among fowl cholera outbreak strains, for reasons yet to be determined. Serial passage of strains in serum and live birds and comparison of a wider collection of avirulent and virulent strains is required to reach a sound conclusion on the effect of host on serum resistance to *P. multocida*. Increase in serum resistance subsequent to serial passage in human serum has been demonstrated in *Neisseria gonorrhoea* strains (Ram *et al.*, 1999). This effect has been demonstrated due to sialic acid modification of gonococcal lipo-oligosaccharide, but a similar phenomenon has not been shown to occur in *P. multocida*. However, a change in the

virulence of *P. multocida* through a serial intravenous passage in turkeys has been reported (Matsumoto & Strain, 1993). It is possible that, through repeated transmissions among birds, a strain may adapt to serum components and grow better than newly introduced strains. However, this cannot fully explain the serum resistance of *P. multocida* to avian sera as the present results have shown that resistant strains are present among non-avian isolates and *P. dagmatis* strains. This suggests the involvement of other determinants of resistance to *P. multocida* to serum bactericidal activity.

Early findings by Griffiths (1974) suggested that RNA is the target for antibody-mediated complement activity to *P. multocida*, but the role of RNA in non-antibody-mediated complement killing of serum-sensitive and serum-resistant strains has not been determined. Subsequently, attempts were made to transform *P. multocida* serum-sensitive strains to serum-resistant strains by cloning with plasmids from serum-resistant strains (Lee & Wooley, 1995). However, the transformants obtained were of intermediate serum resistance compared with a serum-resistant field isolate. This indicated involvement of other factors in serum resistance of *P. multocida*. Hansen & Hirsh (1989) showed that the hyaluronic acid capsule was responsible for serum resistance among capsular type A strains. Subsequent investigations by Diallo & Frost (2000) showed that seven strains remained serum resistant while three remained serum sensitive after treatment with hyaluronidase. In the present study, the protective effect of the capsule to serum complement activity was not confirmed, which underlines the fact that the serum resistance of *P. multocida* is multifactorial.

Although the outbreak clone P-40605, which is serum resistant, caused more severe lesions in experimentally infected chickens, serum resistance cannot be concluded to be the only determinant of virulence of *P. multocida*. Lesions caused by the serum-sensitive strain KC 14Hpg were more severe than the serum-resistant strain MC 6BA, as well as the sensitive strain Mamo 2. Strain MC 6BA was not recovered from trachea of the chickens 24 h after infection, whereas the remaining strains were re-isolated (Table 3). However, it was only the outbreak clone that remained in the trachea for up to 14 days, which indicated that the clone was able to colonize in the trachea mucosa for a long time. These findings indicate that serum resistance coupled with the ability to colonize the trachea may influence virulence of *P. multocida* strains to chickens. Glorioso *et al.* (1982) demonstrated that the presence of adhesion factors on the cell surface were responsible for colonization of the pharyngeal mucosa by *P. multocida* strains that cause respiratory tract infections in rabbits. Further investigations on the combined influence of adhesion factors and serum resistance might increase the understanding of virulence of *P. multocida*.

In conclusion, this study has shown that resistance of *P. multocida* to serum activity occurs among strains from different hosts. Turkey serum was shown to be less inhibitory than other sera investigated. Although the serum-resistant strains are not clonal, strains with the same genotype have the same serum activity, suggesting that the trait is heterogeneous within the species *P. multocida*. The findings also underline that *P. multocida* serum resistance is multifactorial.

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RÉSUMÉ

Résistance de *Pasteurella multocida* dans les sérums aviaires et porcins et etude comparative chez le poulet de la virulence des souches sélectionnées résistantes ou sensibles

La croissance de *Pasteurella multocida*, dans du sérum de différentes espèces poule, dinde, cane et porc, a été comparée et les souches ainsi sélectionnées, résistantes ou sensibles, ont été inoculées à des futures pondeuses âgées de 18 semaines. Quatre-vingt-sept souches de *Pasteurella* spp isolées du terrain et neuf souches de référence représentant différents clones définis par l'analyse des profils de restriction enzymatique (REA) ont fait l'objet de cette étude. L'activité des sérums a été mesurée par la densité optique (OD) des sérums après inoculation et incubation à 41°C pour ceux de poule, dinde et cane et à 39°C pour celui de porc. L'activité des sérums a été mesurée en comparant les résultats obtenus à ceux des souches reconnues comme résistante (P-1059) et sensibles (CU vaccinale) et les souches ont été classées résistantes (R), moyennement résistantes (M) et sensibles (S). Les souches présentant le même type REA ont présenté des courbes de croissance identiques et les mêmes valeurs maximales d'OD quand elles ont été testées dans le sérum des mêmes espèces. Le sérum de dinde s'est révélé être le moins inhibiteur vis-à-vis d'une grande variété de souches de *P. multocida* comparé aux sérums de poule, cane et porc. Des souches résistantes ont été mises en évidence aussi bien parmi celles isolées des espèces aviaires que mammifères. Parmi les souches aviaires, la proportion de souches résistantes a été plus importante chez celles isolées de cas pathologiques que celles isolées de porteurs sains. Le traitement à la hyaluronidase pour éliminer la capsule de certaines souches n'a pas entraîné de changement en ce qui concerne l'activité du sérum. Les lésions les plus importantes chez les poulets infectés expérimentalement ont été induites par des souches résistantes, cependant des lésions ont été également observées chez des poulets infectés par des souches sensibles, indiquant l'implication de facteurs multiples dans la virulence de *P. multocida*. Des investigations supplémentaires sur la résistance induite par le sérum sont proposées avant d'établir un rapport avec les facteurs liés à la bactérie ou à l'hôte et responsables du développement du choléra aviaire.

ZUSAMMENFASSUNG

Serumresistenz von *Pasteurella multocida* in Geflügel- und Schweineseren und vergleichende Virulenzuntersuchungen ausgesuchter serumempfindlicher und -resistenter Stämme bei Hühnern

Das Wachstum von *Pasteurella multocida* und verwandten Spezies im Serum von Huhn, Pute, Ente und Schwein wurde verglichen, und ausgesuchte serumresistente und serumempfindliche Stämme wurden in 18 Wochen alte Legehennen inokuliert. Siebenundachtzig Feldstämme von *Pasteurella* spp. und neun Referenzstämme, die verschiedene, durch Restriktionsendonuklease-Analyse (REA)-Profile definierte Klone darstellten, wurden in dieser Studie verwendet. Die Serumaktivität wurde an den Veränderungen der optischen Dichte (OD) des Serums nach der Inokulation und Inkubation bei 41°C (Vogelserum) bzw. 39°C (Schweineserum) gemessen. Die Serumaktivität wurde durch Vergleich mit zuvor ermittelten serumresistenten (P-1059) und serumempfindlichen (CU-Vakzine) Stämmen beurteilt, und die Feldstämme wurden in stark serumresistent (R), mäßig serumresistent (M) und serumempfindlich (S) eingeteilt. Stämme vom gleichen REA-Typ hatten bei der Untersuchung in Serum der gleichen Wirtsart identische Wachstumskurven und die gleichen maximalen OD-Werte. Es wurde festgestellt, dass Putenserum gegenüber einer großen Auswahl von *P. multocida*-Stämmen weniger hemmend war als Hühner-, Enten- und Schweineseren. Serumresistente Stämme wurden sowohl unter Vogel- als auch Säugerstämmen nachgewiesen. Unter den Vogelstämmen war der Anteil serumresistenter Stämme bei den Stämmen von Krankheitsausbrüchen höher als bei Stämmen von offenbar gesunden Keimträgern. Die Entfernung der Kapsel von ausgesuchten Stämmen durch Behandlung mit Hyaluronidase veränderte die Serumaktivität nicht. Die stärksten pathologischen Veränderungen bei experimentell infizierten Hühnern wurden durch einen serumresistenten Stamm hervorgerufen, doch wurden Veränderungen auch bei Hühnern gefunden, die mit serumempfindlichen Stämmen infiziert waren, was auf die Beteiligung mehrerer Faktoren an der Virulenz von *P. multocida* hindeutet. Weitere Untersuchungen über die Serumresistenz sind angezeigt, um einen Zusammenhang mit anderen, für die Entwicklung der Hühnercholera verantwortlichen Wirtsfaktoren und bakteriellen Faktoren zu finden.

RESUMEN

Resistencia sérica de *Pasteurella multocida* en sueros porcinos y aviares e investigaciones comparadas de la virulencia de cepas seleccionadas resistentes o sensibles al suero en pollos

Se comparó el crecimiento en suero de *Pasteurella multocida* y de especies relacionadas en sueros de pollo, pavo, patos y cerdo y se seleccionaron cepas suero-resistentes y suero-sensibles que fueron inoculadas en ponedoras de 18 semanas de edad. Para este estudio se utilizaron ochenta y siete cepas de campo de *Pasteurella* spp. y nueve cepas de referencia que representaban diferentes clones definidos por los perfiles de análisis de endonucleasas de restricción (REA). Se midió la actividad del suero por los cambios en la densidad óptica (OD) del suero tras la inoculación e incubación a 41°C en el caso de los sueros de pollo, pavo y pato y a 39°C en el caso del suero de cerdo. La actividad del suero fue evaluada por comparación con cepas suero-resistentes (P-1059) y suero-sensibles (vacuna CU) previamente determinadas y se clasificaron como cepas altamente suero-resistentes (R), moderadamente suero-resistentes (M) y suero-sensibles (S). Se detectó que las cepas con el mismo patrón de REA presntaban una

curva de crecimiento idéntica y los mismos valores de OD máximos al ser probadas en sueros de la misma especie. El suero de pavo resultó ser menos inhibitorio a un amplio rango de cepas de *P.multocida* que el suero de pollos, patos y cerdos. Se encontraron cepas suero-resistentes entre las cepas aviares y de mamíferos. Entre las cepas aviares la proporción de cepas suero-resistentes fue mayor en cepas epidémicas que en cepas provenientes de portadores aparentemente sanos. La eliminación de la cápsula de algunas cepas seleccionadas mediante tratamiento con hialuronidasa no cambió la actividad del suero. Las lesiones más severas en pollos infectados experimentalmente fueron producidas por cepas suero-resistentes, pero también se observaron lesiones en los pollos infectados con cepas suero-sensibles, lo que indica que que existen múltiples factores involucrados en la virulencia de *P.multocida*. Se indican futuros estudios sobre la resistencia en suero para relacionar otros factores bacterianos o del huésped responsables en el desarrollo del cólera aviar.