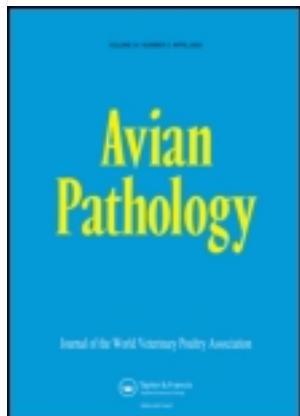


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Time-course investigation of infection with a low virulent *Pasteurella multocida* strain in normal and immune-suppressed 12-week-old free-range chickens

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Twelve-week-old indigenous chickens, either immune-suppressed using dexamethasone (IS) or non-immune-suppressed (NIS), were challenged with a low virulent strain, *Pasteurella multocida* strain NCTC 10322^T, and developed clinical signs and pathological lesions typical of chronic fowl cholera. NIS birds demonstrated much more severe signs of fowl cholera than IS birds. With few exceptions, signs recorded in IS and NIS birds were of the same types, but significantly milder in the IS birds, indicating that immune suppression does not change the course of infection but rather the severity of signs in fowl cholera. *P. multocida* signals by fluorescent *in situ* hybridization (FISH) were observed between 1 h and 14 days in the lungs, trachea, air sacs, liver, spleen, bursa of Fabricius and caecal tonsils, while signals from other organs mostly were observed after 24 h. More organs had FISH signals in NIS birds than in IS birds and at higher frequency per organ. Many organs were positive by FISH even 14 days post infection, and it is suggested that these organs may be likely places for long-term carriage of *P. multocida* following infection. The present study has demonstrated the spread of *P. multocida* in different tissues in chickens and distribution of lesions associated with chronic fowl cholera, and pointed to a decrease of pathology in IS birds. Since dexamethasone mostly affects heterophils, the study suggests that these cells play a role in the development of lesions associated with chronic fowl cholera in chickens.

Introduction

Pasteurella multocida subspecies *multocida* of capsular type A is the cause of different diseases in animals, including fowl cholera in chickens (Christensen & Bisgaard, 2000). This is a severe, systemic condition with sudden onset of clinical signs and high mortality (Glisson *et al.*, 2008). However, in certain production systems, such as the common free-range scavenging poultry production systems in developing countries, the bacterium may be endemic present (Muhaiwa *et al.*, 2001). Under such circumstances, outbreaks of acute fulminating disease are rare, and infected birds show more chronic clinical signs (Mbutia *et al.*, 2008).

During fowl cholera infections, *P. multocida* is believed to enter primarily through the lungs (Christensen & Bisgaard, 2000), even though it has also been shown to invade the intestinal wall in an *in vivo* chicken loop model (Christensen *et al.*, 2002). The bacterium probably adheres to the epithelial lining and subsequently associates with resident macrophages (Matsumoto *et al.*, 1991), and, through a yet to be understood mechanism, it translocates to the bloodstream and from there to internal organs. Polymorphonuclear neutrophils (PMNs) (heterophils) are considered the first line of defence

against the bacterium, but the role of this cell type seems dual, since selective removal of these have been shown to diminish pathological changes (Bojesen *et al.*, 2004). Bacteria have been reported to be detectable in the blood as soon as 6 to 12 h post infection (p.i.) (Rhodes & Rimler, 1990). Death is believed to be caused by endotoxic shock (Harper *et al.*, 2006) and important virulence genes of the bacterium include those encoding the lipopolysaccharide, capsule, fimbriae and other adhesions, in addition to iron-sequestering proteins and a number of outer membrane proteins (Boyce & Adler, 2006; Harper *et al.*, 2006).

Contrary to the detailed knowledge on acute fowl cholera, few reports on the progress of disease in the chronic form are available, including information on the spread of *P. multocida* organisms from the point of infection into the various organs of chickens. We have recently shown that the pig-derived reference strain NCTC 10322^T causes chronic fowl cholera when used to challenge indigenous chickens in Kenya, and further we demonstrated that the age at which these birds are most susceptible with this form of fowl cholera differs from the previously reported age (Mbutia *et al.*, 2008).

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In the current study, we have used this strain to investigate the progress of fowl cholera and the spread of bacteria in a time-course study of chronic fowl cholera in challenged indigenous birds.

As mentioned above, chronic fowl cholera seems to be commonly encountered in low-input production systems, where chickens are subjected to different types of stressful conditions and may suffer malnutrition (Aini, 1999). Such birds may show sub-optimal immune reactions. It has previously been shown that birds which have been manipulated to have a less efficient immune response, especially with respect to the cellular immune system, show a decrease in clinical signs when challenged with *P. multocida* (Bojesen *et al.*, 2004). Since immune suppression influences the outcome of disease and may be a decisive factor in low-input production systems, we performed the time-course study with both immune-suppressed (IS) and non-immune-suppressed (NIS) birds.

Materials and Methods

Experimental chickens. A total of 78 indigenous chickens, 12 weeks old, were used in this experiment. Such birds are of an undefined genetic background with an overall low potential for production. However, ecotypes defined according to phenotypic traits cluster on a genotype defined by microsatellite typing (MSoffe *et al.*, 2001), indicating a degree of selection. Some birds were bought at the age of 1 day old from indigenous flocks in Nairobi, Kenya, while others were hatched from indigenous chicken eggs, also purchased locally.

Bacteria used to infect chickens. *P. multocida* type strain (NCTC 10322^T) maintained on Dorset egg agar was used in the present study. It was spread onto blood agar with 5% citrated calf blood, incubated aerobically at 37°C for 24 h, to check for purity prior to preparation of the inoculums as described previously (Mbuthia *et al.*, 2008).

Immunosuppression of chickens. Birds to be immune-suppressed were injected intramuscularly using dexamethasone (Agar Holland, Soest, The Netherlands) 4 mg/kg body weight per day for 6 days prior to experimental infection, following the protocol of Corrier *et al.* (1991).

Post-mortem examination. Birds were killed by cervical dislocation and post-mortem examination was performed as described by Bermudez & Stewart-Brown (2003). The dead birds were opened aseptically, tissues and organs observed individually, and gross lesions noted and recorded. The severity of the lesions was scored as mild, moderate or severe as done previously (Shivaprasad & Droual, 2002). For microscopic lesions, a minimum of six sections of a particular organ per chicken were examined. They were scored on the basis of – (minus = no lesions), 1 (minimal to mild), 2 (moderate) and 3 (severe). If most sections of the particular organ in one group exhibited minimal to mild lesions, they were given a score of 1, while sections without minimal lesions were recorded as – (minus).

Histological examination: fixation and sectioning. The tissue samples were kept fully immersed in labelled bottles containing 10% formalin solution for 24 h. They were then transferred into 70% alcohol, where they remained until trimming was done. The fixed tissues were manually trimmed to a thickness of 2 to 3 mm and processed as paraffin-embedded sections, sectioned to 3 to 5 µm thickness, placed on microscope slides, stained using haematoxylin and eosin, mounted in destrene 80, dibutylphthalate and xylene, and dried before examination under the microscope.

Fluorescent *in situ* hybridization technique. Paraffin-embedded tissues were mounted on super-frost plus slides (Menzel-Gläser, Braunschweig, Germany) and processed for the fluorescent *in situ* hybridization (FISH) test as described previously (Mbuthia *et al.*, 2001).

Bacterial culture. Swabs were placed in 2 ml sterile phosphate-buffered physiological saline and thoroughly vortexed. Tissue samples (1 to 5 g) were macerated in 2 ml sterile phosphate-buffered physiological saline. From this, streaks were made on blood agar (CM55; Oxoid Ltd, Basingstoke, UK), which were incubated aerobically at 37°C for 24 h, for initial culture. Then 0.1 ml of the remaining contents was inoculated into *Pasteurella*-free 21-day-old Balb/C mice by the intraperitoneal route, as described by Muhairwa *et al.* (2001), to improve the bacterial recovery rate from these samples. Prior to this, five of the mice from the colony were killed, screened, and certified free of *Pasteurella* spp. Inoculated mice were observed for up to 48 h, after which the surviving mice were sacrificed. Severely sick animals were humanely sacrificed prior to this and processed. The mice were dissected, and the livers and spleens removed aseptically and macerated. The macerated material was streaked onto blood agar and incubated aerobically at 37°C for 24 h.

Bacterial colonies from initial swabs and mouse organs morphologically resembling those of *P. multocida* were sub-cultured on blood agar. The *P. multocida* identity was confirmed following the procedures described elsewhere (Bisgaard & Mutters, 1986).

Experimental procedure and clinical observations. Experimental birds were wing tagged with a number, then allocated randomly into infected and control groups and into IS chickens and NIS chickens. The birds were separated and put in the experimental rooms 48 h prior to inoculation. They were screened for *P. multocida* organisms before experimental inoculation by examination of cloacal and oropharyngeal swabs.

For the experiment on NIS chickens, 34 chickens were used: 24 were infected with *P. multocida* organisms and 10 were used as controls. For the experiment on IS chickens, 44 chickens were used: 24 IS birds were infected with *P. multocida* organisms, while the controls consisted of 10 IS and 10 NIS birds.

Each bird in the challenge groups was inoculated intratracheally with 0.5 ml brain heart infusion broth culture containing 1.2 to 1.9×10^8 *P. multocida* organisms, while the controls were inoculated with 0.5 ml sterile brain heart infusion broth medium. The infected and control birds were housed in different rooms located in different houses away from each other. Biosafety measures were maintained during the entire period of the study.

Birds were observed daily for clinical signs by the same person throughout the study period. Initial observations were made without disturbing the birds, and later a close examination and cloacal temperature measurement was carried out while holding each bird. Cloacal temperature below 41.5°C was taken as normal, 41.6°C as mild, around 42.0°C as moderate, and that of over 42.0°C was taken as severe fever.

Two birds, randomly selected from each experimental group, were sacrificed at specified times, namely: hours 0, 1, 3, 6, 12 and 24 and days 2, 3, 5, 7, 10 and 14, post inoculation. Hour 0 was achieved by inoculating the birds and immediately sacrificing them within 5 min. To avoid possible cross-infection, the daily sacrifices started with birds in the control houses before proceeding to the infected houses.

Post-mortem examination was done on the sacrificed birds and swabs and tissue samples were taken aseptically for *P. multocida* re-isolation. Part of the tissue samples were removed and immediately immersed in formalin, where they were kept for histological and FISH test processing.

Statistical analysis. Data obtained during the present study were analysed using the analysis of variance procedures of the statistical analysis systems (SAS Institute, 1996) and the chi-squared test.

Results

No clinical signs, no *P. multocida* re-isolation and no microscopic or gross pathological changes were observed in the control groups.

The IS chickens showed the same types of clinical signs as NIS birds, except for spontaneous tracheal rales, diarrhoea and cyanosis, but at a significantly lower frequency. IS chickens showed a total of 179 clinical

signs and NIS birds a total of 372 signs ($P < 0.05$). The difference was not equally distributed between different organ systems (Table 1).

Depression, ruffled feathers and fever, which are signs of a stage of general infection, combined with signs from the airways such as sneezing, nasal discharges, dyspnoea, head scratching, and mouth discharges, were more common in NIS birds than IS birds, while those with nervous tics were more common in IS birds than NIS birds ($P < 0.05$). Spontaneous rales, diarrhoea, and cyanosis were recorded in the NIS birds only (Table 1). Not only did NIS birds show more clinical signs, the sign were also seen earlier in NIS compared with IS birds, with the majority of the signs recorded between the 1st and 10th days for NIS birds and between the 3rd and 6th days for IS birds (data not shown).

As seen from Supplementary Figure 1, gross lesions were observed on 23 and 22 of the 24 organs examined in NIS and IS birds, respectively. The preen gland (NIS birds) and the preen gland and adrenal gland (IS birds) were negative for the whole of the study period. Lesions were most frequently observed in the lung (NIS birds, 83.3%; IS birds, 75%) and air sacs (NIS birds, 66.7%; IS birds, 58.3%) in both groups, followed by the trachea, liver and spleen (58.3%) in NIS birds and the liver (58.3%), spleen (50%) and kidney (41.7%) in IS birds (Table 1). The number of gross lesions peaked between the 3rd and 24th h in NIS birds and between the 3rd and 12th to 24th h in IS birds, whereafter it decreased gradually to days 10 and 7 p.i. in NIS and IS birds, respectively (Figure 1). Typically, lesions were observed at later time points in NIS birds compared with the same organs in IS birds (Figure 1).

Gross inflammatory lesions/exudates were due to circulatory disturbances, degeneration and necrosis of the affected tissues. Very few lesions were observed at the end of the 2-week study period, except remnants of fibrin and fibrosis on some organs, a possible indication of recovery. Lesions became limited to the respiratory

tissues and liver, spleen and caecal tonsils on the 3rd and 5th day and to the lungs after day 7 in NIS chickens. Only the lungs had lesions after day 5 and no lesions were observed in the 2nd week p.i. on the organs of IS birds (Figure 1).

On microscopic examination, all NIS organs and 20/24 IS organs had microscopic lesions/exudates. Lesions were observed over the full study period, except at 0 h and day 14 p.i. (Supplementary Figure 1). The pancreas, sciatic nerve, preen gland, and pectoral muscle were negative in IS birds. Lesions were frequently observed in the lungs (NIS birds, 83.3%; IS birds, 75%), air sacs (NIS birds, 66.7%; IS birds, 75%), trachea (NIS birds, 75%; IS birds, 58.3%), liver (NIS birds, 66.7%; IS birds, 58.3%) and spleen (NIS birds, 66.7%; IS birds, 50%) in both groups (Table 1). At the 12th hour p.i., all 24 NIS organs had showed pathology. The reproductive organ and the pectoral muscle were the last to be affected. In the IS birds the Harderian gland was the last to show a lesion on the 2nd day p.i. These most commonly observed lesions in both groups were congestion, oedema (in tissues and around blood vessels), haemorrhages, emphysema and atelectasis where granulomas caused obstruction, resulting in localized overinflation in air capillaries and ruptured air sacs with some subcutaneous emphysema, fibrin deposition, infiltration of tissues by heterophils, mononuclear cells and giant cells, and granuloma formation (Figure 2). Lesions were mostly mild (NIS birds, 108 and IS birds, 56). Severe lesions were observed in the respiratory organs (lung, air sacs, trachea), and spleen at 3 h to day 2 day in NIS birds, and in the respiratory organs (3 to 24 h), spleen, liver, and heart at 6 to 12 h p.i. in IS birds (data not shown).

Using the FISH technique, all NIS and 21/24 IS organs had *P. multocida* FISH signals at one or more time points of the study period (Supplementary Figure 1). A typical signal is shown in Figure 3. The sciatic nerve, comb and preen gland were negative in IS birds. The *P. multocida* FISH signals were frequently

Table 1. Comparison of clinical and pathological signs expressed in IS and NIS indigenous chickens infected with *P. multocida*.

Clinical sign	Percentage of samples positive		Organ affected	Percentage of samples positive by histopathological examination		Percentage of samples positive by gross pathological examination	
	IS	NIS		IS	NIS	IS	NIS
Depression	33	79 ^a	Lung	83	83	75	75
Nervous tics	30	13 ^a	Trachea	75	58	58	42
Ruffled feathers	24	74 ^a	Heart	75	33	42	33
Sneezing	25	36 ^a	Air sac	67	75	67	58
Ataxia	14	14	Spleen	67	50	58	50
Nasal discharges	13	34 ^a	Liver	67	59	38	58
Dyspnoea	12	16 ^a	Proventriculus	58	33	17	8
Mouth discharges	1	6 ^a	Conjunctiva	50	17	33	33
Spontaneous rales	–	8	Large intestine	50	17	33	16
Diarrhoea	–	14	Thymus	42	25	33	26
Cyanosis	–	3	Brain	42	33	25	25
Fever	20	60 ^a	Bursa of Fabricia	42	58	33	17
Head scratching	5	15 ^a	Caecal tonsils	42	25	50	8
Total signs	179	372 ^a		141 (108/16/7) ^b	83 (56/19/8) ^b	100 (68/23/9) ^b	81 (61/10/6) ^b

–, no clinical sign seen. ^aStatistically different ($P < 0.05$) from the corresponding group of chickens. The results for histopathology and gross pathology only show results for the most commonly affected organs. ^bNumbers in parentheses show the distribution between signs characterized as mild, moderate and severe.

(a)

Organs	Time points (hour=h;day=d)													
	0 h	1 h	3 h	6 h	12 h	24 h	2 d	3 d	5 d	7 d	10 d	14 d		
Lungs - NIS														
Lungs - IS														
Airsac - NIS														
Airsac - IS														
Liver - NIS														
Liver - IS														
Trachea - NIS														
Trachea - IS														
Heart - NIS														
Heart - IS														
Conjunctiva - NIS														
Conjunctiva - IS														
Thymus - NIS														
Thymus - IS														
Brain - NIS														
Brain - IS														
Bursa of Fabricia - NIS														
Bursa of Fabricia - IS														

(b)

Organs	Time points (hour=h;day=d)													
	0 h	1 h	3 h	6 h	12 h	24 h	2 d	3 d	5 d	7 d	10 d	14 d		
Lungs - NIS														
Lungs - IS														
Airsac - NIS														
Airsac - IS														
Liver - NIS														
Liver - IS														
Trachea - NIS														
Trachea - IS														
Heart - NIS														
Heart - IS														
Conjunctiva - NIS														
Conjunctiva - IS														
Thymus - NIS														
Thymus - IS														
Brain - NIS														
Brain - IS														
Bursa of Fabricia - NIS														
Bursa of Fabricia - IS														

(c)

Organs	Time points (hour=h;day=d)													
	0 h	1 h	3 h	6 h	12 h	24 h	2 d	3 d	5 d	7 d	10 d	14 d		
Lungs - NIS														
Lungs - IS														
Airsac - NIS														
Airsac - IS														
Liver - NIS														
Liver - IS														
Trachea - NIS														
Trachea - IS														
Heart - NIS														
Heart - IS														
Conjunctiva - NIS														
Conjunctiva - IS														
Thymus - NIS														
Thymus - IS														
Brain - NIS														
Brain - IS														
Bursa of Fabricia - NIS														
Bursa of Fabricia - IS														

(d)

Organs	Time points (hour=h;day=d)													
	0 h	1 h	3 h	6 h	12 h	24 h	2 d	3 d	5 d	7 d	10 d	14 d		
Lungs - NIS														
Lungs - IS														
Airsac - NIS														
Airsac - IS														
Liver - NIS														
Liver - IS														
Trachea - NIS														
Trachea - IS														
Heart - NIS														
Heart - IS														
Conjunctiva - NIS														
Conjunctiva - IS														
Thymus - NIS														
Thymus - IS														
Brain - NIS														
Brain - IS														
Bursa of Fabricia - NIS														
Bursa of Fabricia - IS														

Figure 1. Clinical and pathological signs of fowl cholera and presence of *P. multocida* in 10 organs of normal (NIS) and immune-suppressed (IS) birds following challenge of 12-week-old-birds. Two birds were killed and examined at each time point. 1a: Record of the time points where gross pathological lesions were observed in the 10 organs most commonly affected. 1b: Record of the time points where microscopic lesions were observed in the same organs as above. 1c: Record of the demonstration of *P. multocida* by the culture technique in the same organs as above. 1d: Record of the demonstration of *P. multocida* by FISH in the same organs as above. Grey boxes ■ show results for NIS birds, dark-shaded boxes ■ show results for IS birds, light-shaded boxes □ indicate that no analysis was performed at this time point, and white boxes □ show that the result was negative at this time point.

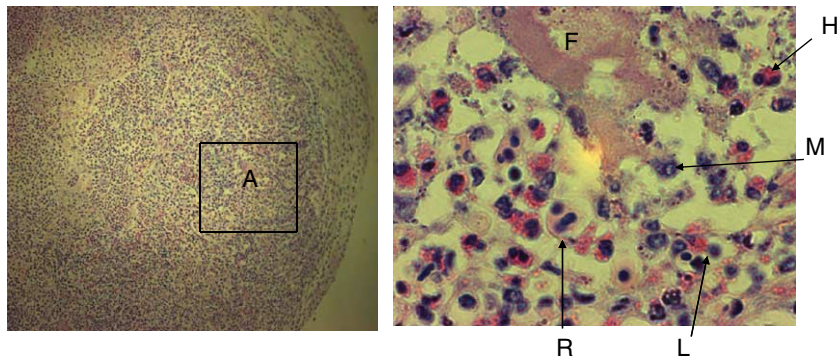


Figure 2. Illustration of the most commonly observed microscopic changes in both IS and NIS birds. 2a: Chicken lung from a NIS bird showing pyogranulomatous fibrinous inflammation ($\times 40$). 2b: High magnification of "2a" ($\times 400$). F, fibrin exudate; H, heterophil; M, macrophage; L, lymphocyte; R, erythrocyte (haematoxylin and eosin stain).

observed in the lungs (NIS birds, 100%; IS birds, 91.7%), air sacs (NIS birds, 75%; IS birds, 66.7%), trachea (NIS birds, 100%; IS birds, 66.7%), and liver (NIS birds, 83.3%; IS birds, 75%). Most FISH signals were observed between 1 and 24 h p.i. (Figure 1).

P. multocida organisms were recovered less frequently by culture method than by FISH (Figure 1 and Supplementary Figure 1). Most bacterial cultures were recovered from the lungs (NIS and IS birds, 58.3% each), trachea (NIS birds, 75%; IS birds, 83.3%), liver (NIS birds, 25%; IS birds, 8.3%), spleen (NIS birds, 50%; IS birds, 8.3%), and caecal tonsils (NIS and IS birds, 25% each). On the 2nd week p.i., there were no bacteria recovered from organs, despite the mouse challenge used to multiply the low number of bacteria (Figure 1).

Discussion

In the present study, the spread of *P. multocida* organisms, the clinical signs, lesions (gross and microscopic), their distribution and bacterial occurrence (FISH and culture) in 24 tissue organs of 12-week-old NIS and IS indigenous chickens were investigated following challenge with a *P. multocida* strain NCTC 10322^T. The strain has previously been shown to cause a chronic form of fowl cholera in the same type of birds with the same challenge dose (Mbutia *et al.*, 2008). The study both allowed us to detail the time course of development of fowl cholera and to determine the differences in pathology and spread of bacteria in IS and NIS chickens. In general, the clinical and pathological signs observed in NIS birds were typical those reported for intermediate to chronic fowl cholera (Christensen & Bisgaard, 2000; Glisson *et al.*, 2003), indicating that the strain used

indeed does induce the chronic form of fowl cholera. However, in addition to signs previously reported, we also observed ataxia, nervous tics and head scratching in these birds. There was often variation between the two birds sacrificed at the same time point in each group with regard to severity and time point for first onset of clinical signs and pathological lesions. This corroborates previous observation of large bird-to-bird variation in chronic fowl cholera (Christensen & Bisgaard, 2000; Glisson *et al.*, 2008). This variation is in striking contrast to acute cases of this disease.

We observed lesions as well as bacteria for up to 14 days in some organs. The relatively long time period where *P. multocida* persisted in many organs, apparently without passing a threshold for toxic concentrations of endotoxin, may be due to a low load of bacteria in the organs. Our study was not set up to quantify bacteria, and further studies are indicated to clarify this point. However, culture and the FISH technique often did not show bacteria even when pathological changes were clear, and this may indicate that the concentration of bacteria is very low.

As part of the study, we compared culture, culture supplemented with enrichment of bacteria in mice and the FISH technique for detection of bacteria in the affected organs. The FISH test results compared very well with the presence of gross and microscopic lesions, while detection of *P. multocida* by culture and FISH were only comparable up to day 3 p.i. (NIS birds) and 24 h (IS birds). Thereafter, the FISH test was found to detect the bacteria better than the other methods. *P. multocida* FISH signals were detected in the preen gland and large intestines, but no bacteria were isolated from these organs. Culture technique especially detecting bacteria in the lungs, trachea and liver, and this may indicate that

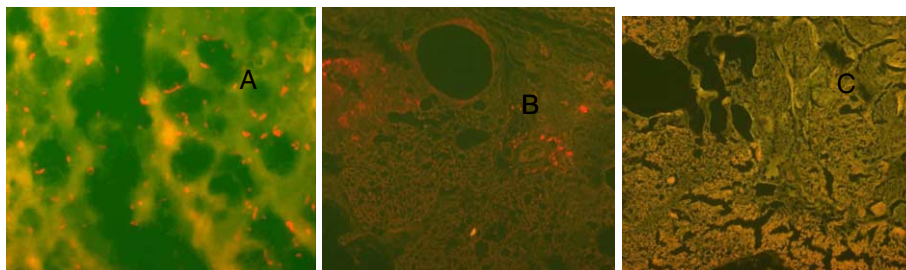


Figure 3. Illustration of FISH signals. The figure shows samples of chicken lungs from NIS birds: (3a) bacteria in air capillaries, (3b) bacteria in pneumonic lesions positive for *P. multocida* on FISH test, and (3c) control lung.

these organs carry the highest bacterial load; however, we did not quantify bacteria in the current study. The low recovery is a general *P. multocida* re-isolation trend, which has also been reported for commercial birds, where recovery after 48 h is usually scanty (Christensen & Bisgaard, 2000; Petersen *et al.*, 2001).

Mouse inoculation has until now been considered the most sensitive method (Christensen & Bisgaard, 2000), for which reason we included it in the present study. However, the use of FISH may eliminate the need for experimental animals in this type of study.

The fact that FISH signals correlated far better with the pathological signs observed suggest that this method is superior to culture for detection of *P. multocida* during this type of infection; however, we cannot exclude the possibility that FISH to a certain degree detects bacteria that are no longer viable. The signal intensity depends on the amount of rRNA present in the ribosomes of the cell (Moter & Göbel, 2000). The stability of ribosomes in *P. multocida* in different growth stages is unknown, but studies of *Escherichia coli* suggest that ribosomes are degraded to 50% when the growth enters from the logarithmic into the stationary phase, and that the ribosomes thereafter are stable for several hours (Pii *et al.*, 2011). Based on this, one should not put too much emphasis on differences in detection by FISH in the early parts of the study, but it seems unlikely that FISH signals will be false positive for more than a couple of hours post death of the bacterium. FISH signals were detected as early as 5 min p.i. in the NIS chickens (trachea and lungs), but not in IS birds. This section sample was taken immediately after the intratracheal challenge, and we believe that the positive lung sample was due to contamination during processing of the slide for microscopic examination.

The IS chickens in general had fewer and less severe lesions in lymphoid-rich tissues (spleen, caecal tonsil), and it appears that immune suppression, in particular, affects the expression of lesions in these organs of the indigenous chickens. The dexamethasone treatment applied for immune suppression in the current study was initially used by Courrier *et al.* (1991) to study the effect of immune suppression on caecal colonization of broiler chickens with *Salmonella*. They found no differences in colonization numbers depending on immune suppression, whether this was done by use of cyclophosphamide, cyclosporin or dexamethasone. Dexamethasone treatment results in increased production of PMNs in the bone marrow of human patients (Dale *et al.*, 1975), but suppresses acute inflammatory responses by preventing neutrophil movement to an inflammatory site (Dale *et al.*, 1974). The mechanism is not fully understood, but by use of a nylon adhesion model as a surrogate for epithelial cells, McGruder *et al.* (1995) did not observe less adhesion by heterophils from dexamethasone-treated compared with normal chickens. Heterophilic cells, the chicken equivalent of PMNs, are supposed to be the first line of defence against *P. multocida* infection (Pedersen *et al.*, 2001). Our results clearly demonstrated that chickens treated with dexamethasone developed fewer clinical signs and less severe pathological changes following infection with *P. multocida*. This indicates that at least a part of the clinical signs in chronic fowl cholera are caused by the immune system, and supposedly the heterophilic response. This result corroborates previous results of an

acute *P. multocida* infection model, in which 5-fluorouracil was used to deplete birds from heterophils (Bojesen *et al.*, 2004), and suggests that more emphasis should be put into identification of the factors of *P. multocida* that stimulate heterophil activation.

The time points at which different organs became positive for bacteria, and the rate at which this happened, allowed us to hypothesize a likely pathogenesis of infection. Following the intratracheal challenge, organs of the airways (trachea, lung, air sacs) quickly became positive. Since we used both *in situ* hybridization and culture, we were able to conclude that an invasion and not just passive colonization happened (Figure 3). Conjunctiva also turned positive within the first hours, but this may possibly be due to spillover from the inoculation. There was a quick spread to the spleen and liver within 1 h post inoculation, and by 3 h post inoculation the heart and a range of other organs were also FISH and/or culture positive. All organs had shown FISH signals between the 1st and 24th h, except the preen gland. This showed that *P. multocida* spread quickly to all body sites, which is a hallmark of per-acute infection seen with highly virulent strains (Christensen & Bisgaard, 2000). This happened both in NIS birds and IS birds. Apparently, the difference in severity of infection observed between highly virulent strains and the low-to-medium virulent strain used in the current study does not related to the speed by which it spreads systemically from the port of entrance.

Some organs were positive by FISH even 14 days p.i. The organs that had been most severely affected and where bacterial cultures were positive with the highest frequency (i.e. those organs that most probably carried the highest load of bacteria) were positive in both NIS and IS birds, and it is possible that they simply remain positive, because the time it takes to clear the infection from a particular site is dependent on the number of bacteria. Late (re)colonization, as judged by FISH, was observed for lymphoid tissue in the caecal tonsils (IS birds) and the bursa of Fabricius (both NIS and IS birds), and it may be that this type of tissue constitutes a site for long-term carriage of *P. multocida* following chronic fowl cholera infection.

The infection of the gastro-intestinal tract tissues, observed in the present study, raises a possibility that this *P. multocida* strain can enter or be shed through it, as suggested by Lee *et al.* (2000) and Muhairwa *et al.* (2001). Indeed, in an intestinal loop infection assay it has been demonstrated that different *P. multocida* strains survive well in the gut (Christensen, 2002); however, confirmation from natural infections still needs to be produced. Infection of the reproductive organs (testis and ovary) observed in the study has not been reported previously, although Bisgaard (1995) was able to isolate the bacterium from the oviducts of some ducks. *P. multocida* organisms shed from the cloaca can, therefore, emanate from the gastro-intestinal tract (bursa of Fabricius), reproductive and urinary systems.

In summary, we have described in detail the time course of chronic infection with fowl cholera in normal and immune-suppressed chickens. In accordance with a previous report on acute fowl cholera (Bojesen *et al.*, 2004), normal birds showed more clinical signs and pathological lesions and for a longer period than immune-suppressed birds. The influence of dexamethasone on the immune system is not fully understood, but

taken together our results indicate that the heterophilic cells and other parts of the cellular immune system contribute to the development of pathological lesions seen in infected birds.

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Caecal tonsils - NIS	G	H	G	1	1	1	1	2	1	1	1	1	H	G	H	1	H	G	H	G	H	G	H	
Caecal tonsils - IS	F	C	F	2	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Large intestines - NIS	G	H	G	1	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Large intestines - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Bursa Fabricius - NIS	G	H	G	H	G	H	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	
Bursa Fabricius - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	G	H	2	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Pancreas - NIS	G	H	G	H	G	H	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Pancreas - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	G	H	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Thymus - NIS	G	H	G	H	1	1	2	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Thymus - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Harderian gland - NIS	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Harderian gland - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Brain - NIS	G	H	G	H	1	1	1	2	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Brain - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Sciatic nerve - NIS	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Sciatic nerve - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Testis/ovary - NIS	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Testis/ovary - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Adrenal - NIS	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Adrenal - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Pectoral - NIS	G	H	G	H	2	1	2	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Pectoral - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Comb - NIS	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Comb - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	1	1	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
Pruning gland - NIS	G	H	G	H	G	H	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
Pruning gland - IS	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C
	G	H	G	H	G	H	1	1	1	1	1	1	G	H	G	H	G	H	G	H	G	H	G	H
	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C	F	C

Supplementary Figure 1 Clinical and pathological signs of fowl cholera and presence of *P. multocida* in normal (NIS) and immune-suppressed (IS) birds following challenge of 12-week-old-birds. Records were made from 24 different organs. At each time point, a record is given for NIS and IS birds for gross pathological changes present (marked G), histopathological changes (marked H), positive for *P. multocida* by FISH (marked F) and positive for *P. multocida* by culture method (marked C). White boxes indicate negative results and grey boxes indicate that no record was made. Numbers in boxes are birds out of two that were found positive.