Emerging *Chlamydia psittaci* infections in chickens and examination of transmission to humans

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Abstract

Chlamydia psittaci and atypical *Chlamydiaceae* infections are (re)-emerging in chickens. We therefore examined the prevalence of *C. psittaci*, atypical *Chlamydiaceae* and their zoonotic transmission on 19 Belgian chicken farms. Atypical *chlamydiaceae* were not detected in chickens but 18 of 19 and 14 of 19 farms were positive for *C. psittaci* by both culture and PCR, respectively. *C. psittaci ompA* genotypes A and D were discovered. None of the examined humans (n= 31) was infected with atypical *Chlamydiaceae*, but 29 (93.5%) and 14 (45%) of them were positive for *C. psittaci* by both culture and PCR, respectively. Genotypes A, D and a mixed infection with genotypes C and D were found. Humans (n = 2) working in the *C. psittaci* negative farm never had respiratory complaints, while 25 of 29 (86.2%) positive farmers, reported yearly medical complaints potentially related to psittacosis. Four of them currently experienced respiratory disease and one of them was being treated with antibiotics. Four farmers (12.5%) mentioned that they had pneumonia after start keeping chickens. Occupational physicians should be aware of emerging *Chlamydiaceae* infections in chickens.

Keywords: *Chlamydia psittaci*, atypical chicken *Chlamydiaceae*, zoonosis, psittacosis, chickens

INTRODUCTION

Chlamydiaceae are gram-negative obligate intracellular bacteria and the species *Chlamydia psittaci* (*C. psittaci*) causes respiratory disease in birds. *C. psittaci* infections could be demonstrated in at least 465 different bird species, spanning 30 different bird orders (Kaleta & Taday, 2003). The symptoms may vary from unapparent to severe, depending on the chlamydial strain, stress condition, age and health status of the avian host. The symptoms in birds include rhinitis, conjunctivitis, nasal discharge, dyspnoea, diarrhoea, polyuria, anorexia, lethargy and dullness (Vanrompay *et al.*, 1995). *C. psittaci* is a well-known zoonotic agent causing psittacosis or parrot-fever in humans. During the last 3 decades, psittacosis outbreaks were reported in the US (Grimes & Wyrick, 1991; Newman *et al.*, 1992), China (Ni *et al.*, 1996), India (Chahota *et al.*, 2000), Australia (Tiong *et al.*, 2007) and European poultry industries (Laroucau *et al.*, 2009; Ryll *et al.*, 1994; Sting *et al.*, 2006; Van Loock *et al.*, 2005a; Vanrompay *et al.*, 1997). Zoonotic transfer occurs through inhalation of contaminated aerosols originated from feathers, fecal material and respiratory tract exudates. Handling the plumage, carcasses and tissues of infected birds and in rare cases, mouth-to-beak contact or biting also possess a zoonotic risk (Beeckman & Vanrompay, 2009). Psittacosis in humans may vary from unapparent to fatal in untreated patients (Kovacova *et al.*, 2006). Symptoms include high fever, chills, headache, myalgia, non-productive coughing and difficult breathing (Beeckman & Vanrompay, 2009).

C. psittaci infections mostly occur on turkey or duck farms. However, *C. psittaci* infections are emerging in European and Asian chickens. Recently, Dickx *et al.*, (2010) examined Belgian broiler breeder, broiler and layer farms by a *C. psittaci* recombinant MOMP-based antibody ELISA (Verminnen *et al.*, 2006) and found 98, 95, and 95% seropositive layers, broilers, and broiler breeders, respectively. Moreover, they demonstrated *C. psittaci* genotype D in the air of chicken hatching chambers and in slaughtered Belgian and French broilers.

Zoonotic transmission to hatchery and abattoir employees did occur (Dickx *et al.*, 2010; Dickx & Vanrompay, 2011), albeit without severe clinical consequences. Recently, Yin *et al*., (2012), proved Hill's-Evans' postulates for *C. psittaci* genotype B and D strains isolated from Belgian and French broilers.

Larouceau *et al.*, (2009) detected a new atypical chlamydial agent in chickens. The atypical chicken *Chlamydiaceae* (ACC) caused apparently no disease in infected chickens, but the detection of ACC coincided with 3 cases of atypical pneumonia in individuals working in a French poultry abattoir. In 2012, ACC have been detected in Australian, German, Greek, Croatian, Slovenian and Chinese chicken flocks (Robertson *et al.*, 2010; Zocevic *et al.*, 2012). Importantly, ACC are not detected with *C. psittaci*-specific molecular tools, rendering the need for an ACC-specific PCR. The zoonotic potential and the exact taxonomic status of ACC have yet to be defined.

The aim of the current study was to examine the presence of *C. psittaci* and ACC on Belgian chicken farms, as well as their zoonotic transmission to farmers.

METHODS

Study concept

We investigated the presence of *C. psittaci* and ACC, as well as their zoonotic transmission, on 19 Belgian chicken farms: 7 broiler breeder (1600 to 50,000 animals), 7 broiler (200 to 150,000 animals) and 5 layer (7000 to 22,000 animals) farms from 4 difference geographical regions (Antwerp, East-Flanders, West-Flanders and Limburg). Only 1/19 farms kept additional birds species (ducks and geese). The study was conducted in the summer of 2012. Participating poultry farms were randomly recruited by phone. A sampling package was brought to each poultry farm and sampling was performed immediately. The package contained a questionnaire designed to assess information on: 1) the farmers' professional and nonprofessional activities, smoking habits, general health status, use of medication, influenza vaccination, allergies, clinical signs potentially related to psittacosis, 2) the chicken breed, hatchery, housing, feeding, health status, medication, mortality and 3) the presence of other animals on the farm. The package also contained rayon-tipped aluminium shafted swabs (Copan, Fiers, Kuurne, Belgium) for pharyngeal sampling of 10 ad random selected chickens and the farmers (max 2 per farm). Sampling of the chickens was performed by one of the researchers. In the mean time, humans sampled themselves (informed consent) while being in their home. Swabs for culture contained 2 ml chlamydia transport medium (Vanrompay *et al.*, 1992) while those for PCR contained 2ml DNA stabilization buffer (Roche, Brussels, Belgium). Packages were transported on ice and stored at -80°C until use.

C. psittaci culture

Culture was performed using Buffalo Green Monkey (BGM) cells, identifying the organism by a direct immunofluorescence staining (IMAGEN™, Oxoid, United Kingdom) at 6 days post-inoculation. *C. psittaci* organisms were identified by using the IMAGEN™ direct immunofluorescence assay (Vanrompay *et al.*, 1994). *C. psittaci* positive cells were monitored using a CX31 fluorescence microscope (600 x, Nikon Eclipse TE2000-E, Japan) and presented by a score ranging from 0 to 5 (Table I).

C. psittaci genotyping and PCR detection of atypical *Chlamydiaceae*

DNA extraction of swabs was performed as described by Wilson *et al.* (1996). Briefly, specimens were centrifuged (13,000 x g), suspended in 198 µl STD buffer (0.01 M Tris-HCl [pH 8.3], 0.05 M KCl, 0.0025 M MgCl2.6H20, 0.5% Tween20) and 2 µl proteinase K (20 mg/ml stock solution; Sigma Chemical Co.). The specimens were incubated at 56°C for one hour and subsequently heated at 100°C for 10 min.

A *C. psittaci* specific nested PCR with internal inhibition control was used (Van Loock *et al.* 2005b). Outer membrane protein A (*ompA*) genotyping was performed by a *C. psittaci* genotype-specific real-time PCR (Geens *et al.*, 2005). The latter PCR distinguishes genotypes A to F and E/B using genotype-specific primers, genotype-specific probes and competitor oligonucleotides. Samples of chickens and humans were also examined for atypical chicken *Chlamydiaceae* by use of a recently developed 16S rRNA-based ACC-specific real-time PCR (Zocevic *et al.*, 2013).

Statistics

Potential zoonotic risk factors were statistically examined using SPSS (Inc., Chicago, Illinois, US). Logistic regression was used to search for non-exposure related risk factor for *Chlamydiaceae* culture/PCR positivity. The model contained data on the acquired information of the questionnaire.

RESULTS

C. psittaci and ACC in chickens

Nineteen of 32 (59%) contacted chicken farms participated, resulting in samples from 190 chickens (10 per farm) and 31 humans (max 2 per farm).

Atypical chicken *Chlamydiaceae* were not detected. 18/19 (94.7%) farms were positive for *C. psittaci* by both culture and nested PCR (Table II). The percentage of culture positive chickens per farm varied from 60 to 100%. *C. psittaci* genotype D was present in 17/18 (94.4%) positive farms, while a genotype A infection was discovered in 1 of 18 positive farms (Table III). Thus, *C. psittaci* was found in broiler breeders, broilers and layers. According to the questionnaire, respiratory symptoms were present in infected broiler breeders (3 of 7 farms; 42.8%), infected broilers (5 of 7 farms; 71.4%) and infected layers (1 of 5 farms; 20%). Mean mortality for infected broiler breeders, broiler and layer farms, was 5.4%, 2.8% and 9.8%, respectively. One of 6 infected broiler breeder, and 2 of 7 infected broiler farms currently used antibiotics (tylosine, Pharmasin®, Eurovet and doxycycline, Soludox®, Eurovet). Nevertheless, we were able to detect viable *C. psittaci*. A high stocking density (number of chickens/m^2) was significantly related to the risk of acquiring chlamydiosis ($p = 0.006$). The negative farm was the only with no poultry farms nearby (<4 km). Plus, it was the only farm with a very long sanitary period (8 weeks), which is the period in between emptying the barn, cleaning, disinfection and restocking (usually 1 to 2 weeks). However, the latter two observations were not significantly related to the risk of chlamydiosis in chickens ($p = 0.08$ and 0.157, respectively). Antibiotics were not used at the moment of sampling.

Zoonotic transmissions

The study population consisted of 11 women and 20 men and the average age was 42 years. Three of 31 farmers (9.6%) were vaccinated against human influenza. None were infected by ACC. However, 29/31 (93.5%) humans were *C. psittaci* positive by both culture and the *C. psittaci*-specific nested PCR. *C. psittaci* genotype D (n=26), genotype A (n=1) and a mixed genotype D plus C infection (n=1), was discovered in farmers. Genotyping revealed no result for one sample. The sample originated from a female employee of a layer farm, which only kept chickens (Table IV). Thus, *C. psittaci* zoonotic transmission was detected on all but one examined chicken farm.

Many *C. psittaci* positives were found, but only 4 of them (13.7%), who were non-smokers and had no allergies, currently experienced respiratory diseases (coughing, n = 3 and/or rhinitis, n = 1; sinusitis, n = 1; severe bronchitis, n = 1). They were all infected with genotype D, and the person with bronchitis was currently treated with Augmentin® (Glaxo Smith Kline), respectively. We informed the farmers and their physicians on the diagnostic results.

Humans (n=2) working in the *C. psittaci* negative farm never had respiratory complaints, while 25 of 29 positive farmers (86.2%), reported yearly medical complaints potentially related to psittacosis (Table IV). Four of 31 farmers (12.5 %) mentioned that they had pneumonia after start keeping chickens (Table IV).

No potential risk factor like age, gender, living in the direct environment of the farm, number of years employed in the sector, daily time in contact with chickens, pet animals, smoking behavior and medical complaints were significantly related with psittacosis.

DISCUSSION

We examined the occurrence of *C. psittaci* on 19 Belgian chicken farms, as well as zoonotic transmissions of these pathogens to farmers as *C. psittaci* is (re)-emerging in chickens. Limited reports from 1960 to 2000 suggest that chickens are less sensitive to *C. psittaci* infections. However, during the last decade, *C. psittaci* was detected and isolated from chickens raised in Australia, Belgium, China, France and Germany (Yang *et al.*, 2007; Gaede *et al.*, 2008; Zhang *et al.*, 2008; Laroucau *et al.*, 2009; Robertson *et al.*, 2010; Zhou *et al.*, 2010; Dickx & Vanrompay, 2011). Recently, Yin *et al.*, (2012), proved Hill's-Evans' postulates for *C. psittaci* genotype B and D strains isolated from Belgian and French broilers. Less is known on *C. psittaci* genotypes infecting chickens. Up to now, genotypes B, C, D, F and E/B have been found in chickens (Gaede *et al.*, 2008; Zhang *et al.*, 2008; Dickx *et al.*, 2010; Zhou *et al.*, 2010; Yin *et al.*, 2012).

C. psittaci is apparently not the only emerging chlamydial pathogen in chickens. Laroucau et al., (2009), discovered a new chlamydial agent in chickens raised in France, designated atypical chicken *Chlamydiaceae* (ACC). Remarkably, ACC positive chickens appeared healthy, but the discovery of ACC coincided with three cases of atypical pneumonia in French poultry workers (Laroucau *et al.*, 2009), warranting the need for epidemiological surveillance in chickens. Since then, ACC has been found in chickens raised in China, Croatia, Germany, Greece and Slovenia (Zocevic *et al.*, 2012). This is why we also included the recently developed ACC-specific real-time PCR in our epidemiological study.

C. psittaci was highly prevalent in chickens and humans. *OmpA* genotyping revealed the presence of genotypes A, C, and especially D. To our knowledge, this is the first time that genotype A, the second time that genotype C, and only the third time that genotype D has been identified in chickens. Genotype A is most often found in *Psittaciformes* (cockatoos, parrots, parakeets, lories) and is frequently being transmitted from pet birds to humans. Genotype A has also been isolated from turkeys and wild birds (Van Loock et al., 2005; Verminnen et al., 2008, Geigenfeind et al., 2011; Kalmar *et al*., 2013). Thus, the pathogen is not restricted to *Psittaciformes* and was probably never noticed before in chickens. However, genotypes B and D seem to be most prevalent in chickens. Genotype D is most often found in turkeys, but recently has been associated with zoonotic transfer from chickens to slaughterhouse employees (Dickx *et al.*, 2010). Genotype C has primarily been isolated from ducks and geese, but has been found once before in chickens, namely in China (Zhang et al., 2008).

Atypical chicken *Chlamydiaceae* were not detected in chickens, suggesting that ACC is currently not widespread in Belgium chicken flocks, at least when compared to *C. psittaci*. However, we cannot exclude the absence of this emerging chlamydial agent in our chicken flocks. Respiratory disease was present, albeit not on all, *C. psittaci* infected farms. Respiratory disease was most frequently present on broiler farms, followed by broiler breeder and layer farms, respectively. Only broiler and broiler breeder farms claimed to use antibiotics (tylosine, Pharmasin®, Eurovet and doxycycline, Soludox®, Eurovet). Antibiotic usage in European poultry decreased the last years (Moulin *et al.*, 2008; BelVet-SAC report 2012; http://www.belvetsac.ugent.be/), but antibiotics are still frequently used without proper diagnosis and among them are the ones being active against *C. psittaci*, with the risk of creating tetracycline resistance as occurred for *Chlamydia suis* (Dugan *et al.,* 2004).

Interestingly, a high stocking density (number of chickens/m^2) was the only risk factor that was positively correlated with the occurrence of *C. psittaci* in chickens. This finding was no surprise, as *C. psittaci* transmission most often occurs from one bird to another bird close by.

As for chickens, ACC were not detected in farmers. However, viable *C. psittaci* were present in 93.5% of the farmers. Genotypes A, C and, as in chickens, especially genotype D were discovered in the farmers. In our study, genotype C (most frequently found in *Anseriformes*; ducks and geese) was not detected in chickens, but we cannot exclude the absence of genotype C on the farm, as only 10 chickens were sampled. Zoonotic transmissions of genotypes A, C and D, and even mixed genotype A, C and D infections in poultry workers, have been observed before by Dickx & Vanrompay (2011), examining employees of a turkey and chicken hatchery. Thus, *C. psittaci* infected chickens present a substantial zoonotic risk. One human sample could not be genotyped, which could indicate the presence of a new genotype. Attempts to grow the strain to a higher bacterial titer for *ompA* sequencing failed.

Humans (n= 2) of the *C. psittaci* negative farm never had respiratory complaints, while 25 of 29 (86.2%) humans, all working in *C. psittaci* positive farms, reported yearly medical complaints potentially related to psittacosis (Table IV). Four (12.5 %) of 31 farmers mentioned in the questionnaire that they had pneumonia after start keeping chickens, which was higher than the yearly rate of 8/1,000 pneumonia cases in Belgium. It is likely that chicken farmers are regularly infected, creating immunity, which protects them against severe disease. However, yearly complaints about fever and respiratory disease were of interest (Table IV). Whether farmers become carriers, clinical consequences and the importance of co-infections with other human respiratory pathogens are unknown.

Preventing avian chlamydiosis in poultry is difficult because of the endemic nature of the bacteria, the long-term survival of the bacteria in organic material, the intermittently shedding and the many asymptomatic carriers (Pelle-Duporte & Gendre, 2001). An all-in, all-out rearing regime, with thorough cleaning and disinfecting between broods is obligatory. *C. psittaci* is highly susceptible to heat and disinfectants (quaternary ammonium compounds, house-hold bleach) but is resistant to drying, acids and alkali (Smith *et al.*, 2005). The access of wild birds to the animals or food should be prevented. Equipment should be regularly cleaned and disinfected when used for several barns at the farm.

Personal protective measures are a good hand hygiene protocol and protective clothing, including gloves and an air filter full-face mask. A transition room should be available where protective clothing may be kept. The two most important collective protective measures are ventilation and cleaning. Natural or mechanical ventilation should try to prevent aerosol accumulation and cross-contamination between the different barns. Even continuous disinfection (although expensive) of the air in the barns could be considered. Education and training are very important to guarantee that the preventive measures are well understood and performed (Deschuyffeleer *et al.,* 2012).

Conclusions

Despite the governments' obligation to assess any biohazard in the workplace, knowledge on *C. psittaci* and especially ACC in chickens is still relatively undeveloped and a specific risk assessment in poultry production has not been composed yet. Many health care providers are not familiar with psittacosis, especially with its occupational and zoonotic character. An occupational physician assigned to modern vertically integrated poultry farming covering the complete poultry production ranging from the feeding mill to processing facilities, could conduct a campaign to raise general awareness and to inform poultry workers on collective and personal protective measures. The occupational physician should address local physicians with a written document as this may lead to an early diagnosis and treatment in poultry workers (Deschuyffeleer *et al.*, 2012). However, most benefit is to be expected from an efficient avian *Chlamydia* vaccine.

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Table I: *C. psittaci* culture scores

Score	Meaning
0	Negative (no EB, no IPC)
1	1-5 EBs
2	6-15 EBs
3	15-25 EBs and/or 1-5 IPCs
4	25-100 EBs and/or 6-15 IPCs
5	1-10 EBs/field and/or 1-5 IPCs/field

EB = elementary body, IPC = inclusion positive cell

Table II: Pharyngeal excretion of viable *C. psittaci* by poultry (n = 10 per farm) and poultry workers (n = 1 or 2 per farm).

	Poultry					Poultry workers		
	Farms	Culture score*		Positive		Culture score*		
Farm Type	Positive/total	Mean ± SD	Range	%within flock	Genotype*	Mean ± SD	Range	Genotype*
Broiler	7/7	1.7 ± 0.6	0-5	94	D (7/7)	1.9 ± 1.4	1-5	D (7/7)
Layer	5/5	1.8 ± 0.5	0-5	94	A (1/5) D (4/5)	2.1 ± 0.9	1-3	A (1/5) D (4/5)
Broiler Breeder	6/7	1.8 ± 0.2	1-4	100	D (6/6)	1.8 ± 0.7	1-3	D (5/6) C,D (1/6)

* Within culture positive farms

Table III: Viable *C. psittaci* and perceived health status in poultry farms

C. psittaci in broiler farms (n = 10 per farm)				Health status broilers (questionnaire)			
Age (weeks)	**Positive (%)**	**Score (Mean ± SD)**	**Genotype**	**Density (#/m^2)**	**Mortality (%)**	**Resp Symp (%broods)**	**AB$_{resp}$ (%broods)**
2	100	2.8 ± 0.8	D	19	2	10	10 (doxy)
< 1	100	2.0 ± 1.2	D	18	3.5	25	0
1	60	0.9 ± 1.0	D	14	3.5	15	0
2-3	100	1.2 ± 0.6	D	20	2.8	10	10 (tylo)
2-3	100	2.0 ± 1.3	D	10*	3	10	0
2-3	100	1.3 ± 0.5	D	20	2	0	0
5	100	1.7 ± 0.9	D	19.5	3	0	0
***C. psittaci* in layer farms (n = 10 per farm)**				**Health status layers (questionnaire)**			
32	100	1.8 ± 1.0	A	7	5	0	0
37	100	1.5 ± 0.8	D	5*	NA	0	0
39	100	2.4 ± 1.0	D	9*	7 - 30	0	0
41	100	2.1 ± 1.3	D	9*	10	10	0
74	70	1.1 ± 1.4	D	9*	4	0	0
***C. psittaci* in broiler breeders farms (n = 10 per farm)**				**Health status broiler breeders (questionnaire)**			
2	100	1.4 ± 0.8	D	10	2	100	0
31	0	0.0 ± 0.0		7	NA	0	0
34	100	2.1 ± 1.2	D	16.5	5 – 10	0	0
42	100	2.0 ± 0.9	D	7.2	10	10	0
48	100	1.8 ± 1.0	D	6.5	9.3	0	0
50	100	1.6 ± 0.5	D	NA	1.5	0	0
50	100	1.9 ± 1.0	D	9	1.2	10	10 (doxy)

*Chickens have the ability be outside
NA: Not Available

Table IV: *C. psittaci*, perceived health status and psittacosis compatible symptoms ([1]once or twice, [2]repeatedly, [3]frequent**) in farm employees.**

Broiler farm employees													
	Viable *C. psittaci*		**Personnel data**			**Current health status**		**Yearly medical complaints**					**Confirmed Pneumonia**
	Score	**Genotype**	**Period**	**Time**	**Aves at home**	**Current symptoms**	**AB treatment**	**Fl**	**Re**	**GI**	**Ey**	**De**	**# years ago**
Male	5	D	27 y	2 h/w	-	-	-	F[1], M[1]	NPC[1]	S[1], D[1]	-	-	-
Male	1	D	20 y	7 h/d	layers	-	-	-	-	-	-	-	-
Male	1	D	2 y	7 h/d	birds	-	-	F[1], M[2]	-	-	-	-	-
Male	1	D	15 y	2 h/d	layers	-	-	F[1], M[1]	NPC[2]	-	-	-	-
Male	1	D	12 y	1h /d	-	-	-	M[2]	-	-	-	R[2]	3 y
Male	2	D	20 y	1 h/d	-	-	-	F[1], M[3]	-	V[1]	E[1]	-	-
Male	1	D	30 y	2 h/d	-	-	-	-	PC[3]	-	-	-	19 y
Female	4	D	25 y	8 h/d	-	-	-	F[3], M[3]	PC[1]	B[1], D[3]	-	R[1]	-
Female	3	D	13 y	3 h/d	-	-	-	F[2], M[2]	NPC[2]	-	-	-	2 y (pleuritis)
Female	1	D	30 y	7 h/d	-	cold	-	every production round a cold at ± 5 weeks					-
Broiler breeder employees													
Male	2	D	15 y	2 h/d	-	-	-	-	NPC[2]	-	-	-	-
Male	1	D	7 y	1 h/d	-	-	-	F[1], M[1]	PC[1]	V[1], S[1], D[1]	-	-	-
Male	2	D	19 y	3 h/d	-	-	-	F[1], M[1]	NPC[1]	S[1]	-	-	-
Male	1	D	4.5 y	8 h/d	-	cold	-	F[1]	NPC[2], B[2]	-	E[2]	-	-
Male	1	D	27 y	4 h/d	-	-	-	-	-	-	-	-	22 y
Male	2	D	25 y	8 h/d	-	-	-	F[2], M[2]	PC[2], B[2]	V[2], S[2], D[2]	E[2]	-	-
Male	0	-	2 y	1 h/d	-	-	-	-	-	-	-	-	-
Male	0	-	17 y	3 h/d	-	-	-	-	-	-	-	-	-
Fe ma	3	D	15 y	2 h/d	-	'allergic feeling'	-	T[3]	NPC[2]	-	-	R[1]	-

	2	D	7 y	2 h/d	-	-	-	F^{1}, M^{2}	PC^{2}	V^{1}, S^{1}, D^{1}	-	R^{1}	-
	2	D	19 y	4 h/d	-	cold	Augmentin (4 wk ago)	-	NPC^{1}	S^{1}	-	-	-
	1	D	27 y	4 h/d	-	-		-	-	-	-	-	-
	3	D + C	30 y	8 h/d	-	-		F^{2}, M^{2}	PC^{2}	V^{2}, S^{2}, D^{1}	-	-	-
Layer farm employees													
Male	3	D	40 y	1 h/d	-	-	-	F^{1}, M^{2}	NPC^{1}, DB^{1}	-	-	-	
	3	D	7 y	5 h/d	-	-	-	M^{2}	NPC^{1}	-	-	-	
	1	D	12 y	0.5 h/w	-	-	-	-	NPC^{3}, Ex^{3}	-	-	-	
	2	D	2 m	0.5 h/w	ducks, geese	Cold	-	F^{1}	-	-	-	-	
	3	A	17 y	3 h/d	-	-	-	F^{2}	-	S^{2}, D^{2}	-	R^{2}	
Female	3	D	24 y	3 h/d	-	-	-	F^{1}, M^{3}	NPC^{1}, B^{1}	-	-	-	
	1	D	23 y	4 h/d	-	-	-	-	-	-	-	-	
	1	NA	3 y	3 h/d	-	-	-	F^{2}, M^{2}	-	-	E^{2}	-	

Fl: Flu like : F, fever; M, myalgia; T, tired-fatigue
Re: Respiratory : NPC or PC, (non) productive cough; B, painful breathing; Ex, morning expectoration
GI: Gastro intestinal : V, vomiting; D, diarrhea; S, stomach ache
Ey: Eye : E, painful eyes
De: Dermatologic : R, non-specific rash
NA: not applicable