Prevalence of etiological agents of selected respiratory infections in chicken and turkey farms in the Czech Republic

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ABSTRACT: The causative agents of respiratory diseases of turkeys represent, primarily in fattening farms, a substantial risk of economic and breeding problems. The purpose of this communication is to provide information on the prevalence of respiratory agents of turkeys and chickens in several fattening and production farms in Southern Moravia. This study was focused on pathogens causing bacterial diseases such as Ornithobacteriosis and Mycoplasmosis, as well as viral rhinotracheitis and laryngotracheitis of poultry. The laboratory diagnosis of these diseases has been performed in our institute since January 2008. We examined 249 samples of turkeys and chickens from a single rearing house and six fattening farms in Southern Moravia. The samples were examined using the PCR or RT-PCR method. The typing of isolates of *Ornithobacterium rhinotracheale* was done using the M13 fingerprinting method. We established the prevalence of pathogens such as *Ornithobacterium rhinotracheale* (ORT), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *avian metapneumovirus* (aMPV) *and laryngotracheitis virus* (ILT) in selected farms.

Keywords: respiratory pathogens; turkey; polymerase chain reaction

Ornithobacteriosis of poultry is a respiratory disease characterized by tracheitis, aerosaculitis and fibrous pneumonia, with an approximate 10% mortality. The course of the disease is frequently aggravated by concurrent infections with other etiological agents and immunosuppression of the host, e.g., by stress. The occurrence of the disease is more frequent in adolescent and adult animals, is accompanied by respiratory signs, and in turkeys also by occasional movement disorders due to the inflammation of ankle and shoulder joints. Eighteen serotypes of *Ornithobacterium rhinotracheale* (A-R) have been described so far (Hafez 2002). Because of the existence of a large number of serotypes autogenous vaccines as preventive antibacterial therapies against bacterial diseases caused by *O. rhinotracheale, E. coli, Pasteurella multocida and Erysipelothrix rhusiopatiae* is used. However, antibacterial therapy is difficult due to the occurrence of resistant strains; therefore, the application of therapeutic drugs is possible only after the results of antimicrobial sensitivity tests. The antibiotics of choice are tetracycline, chlortetracycline and amoxiciline. High seroprevalences of *O. rhinotracheale* infection have been recorded in some countries in Europe and Asia, especially in Germany 26% (Hafez and Sting 1996), in Turkey 62.5% (Turan and Ak 2002) and in Iran 44.2% (Allymehr 2006), mostly in broiler flocks.

Avian mycoplasmosis is a disease of poultry and turkeys that is widespread in commercial farms worldwide. *M. gallisepticum* and *M. synoviae* in chickens and turkeys represent pathogenic agents with variable clinical manifestations. Disease is manifested by chronic respiratory signs in chickens, by infectious sinusitis and aerosaculitis in turkeys and synovitis in chickens and young turkeys. Mycoplasmosis often complicates respiratory diseases with other etiologies. Infection spreads in the flock slowly and persists for a long time. Latent infection is manifested primarily by low weight gains, feed reception and reduced egg laying. Clinical symptoms of the disease include thick mucus or septic discharge from the nose, conjunctivitis and swelling of infraorbital cavities in turkeys. CNS disorders develop occasionally. Infectious synovitis caused by a strain of *Mycoplasma synoviae* is an acute to chronic infectious disease of gallinaceous poultry affecting the synovial membrane of joints and peritenons; sometimes clinical signs are manifested in the upper respiratory tract. Pheasants and geese are also susceptible to infection, while the infection of ducks and other avian species has not yet been described (Kleven 1998). Affected turkeys and chickens have a pale crest due to anaemia, suffer from diarrhoea, motility disorders related mainly to the inflammation of the ankle joint, and are cachectic and dehydrated. Mortality is low if other concurrent infections are absent (1–10%).

The eradication of avian metapneumovirus is based on the serologic elimination of infectious carriers, antibiotic programmes, and vaccination. Currently, vaccines such as Gallivac (live attenuated vaccine), Poulvac and Talovac (inactivated vaccine) are registered and used in the Czech Republic. *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are still detected on turkey and chicken farms. Mycoplasma infections are distributed worldwide. The prevalence has increased in Holland, especially of *M. synoviae*, and in herds of commercial layers it has risen by 73% and in meat turkey by 16% (Feberwee et al. 2008). The prevalence of *M. synoviae* was monitored using the PCR method in herds of laying hens (75%) in Germany, however, PCR analysis failed to detect *M. gallisepticum* in any of the collected samples. (Kohn et al. 2009).

Avian metapneumovirus (aMPV) is an acute viral disease that was first described in turkeys in South Africa (Buys and Du Preez 1980). The virus was later isolated in Great Britain and characterized

as avian pneumovirus (Cavanagh and Barret 1988), and was classified in the genus *Metapneumovirus* (Pringle 1998), family *Paramyxoviridae.* After its first appearance in South Africa in 1978 and then in France and the UK, isolates belonging to different subgroups of avian pneumovirus mainly A or B, were reported in Hungary, Italy, the Netherlands, Spain and in Israel, Mexico, Morocco, Jordan, Brazil and Japan (Cook 2000; Hafez et al. 2000). While the A and B of aPMV types have reported in Europe (Hafez et al. 2000), subtypes C and D have been reported only in the USA and France (Seal 2000; Toquin et al. 2000). The most susceptible virus hosts are domestic fowl (swollen head syndrome) and turkeys of all ages. Of other avian species, only pheasants and guinea fowl are susceptible (Gough et al. 1988), and may play a role in virus transmission. Clinical symptoms are variable, and include sneezing, snuffling, foamy discharge from the nose, breathing issues, conjunctivitis, swelling of infraorbital sinuses and submandibular oedema. Serologically detected subclinical infections are frequent. Respiratory signs in breeding turkeys can be less pronounced, and occasionally include lower egg laying, decolorizing of egg shells, lower hatching, or lower semination of batch eggs (Cook 2000). Secondary bacterial infections (chlamydia, mycoplasmas) often cause hepatitis and splenitis, more severe courses of the disease include pneumonia and inflammation of air sacs (Hafez 1998). Several types of attenuated or inactivated vaccines are distributed in the Czech Republic.

Infectious laryngotracheitis virus (ILTV) causes peracute to acute disease of chickens, turkeys, and pheasants. The ILT virus belongs to the family *Herpesviridae,* subfamily *Alfaherpesvirinae,* genus *Iltovirus.* The disease is manifested by difficulty in breathing, gasping and expectoration of bloody mucus. Conjunctivitis to panophtalmitis is also frequent, worsened mainly by environmental factors, namely in winter, when the respiratory tract is irritated by other infectious agents. The virus is antigenically uniform, field strains differ in virulence only (Chacon and Ferreira 2008). As in all herpesviruses in infected or vaccinated animals lifelong latent infection is established. In the risk area for breeding and egg production flocks vaccination is still widely practiced, using attenuated virus vaccine. This protects birds against disease, but not against infection with virulent virus or development of latent carrier status for either the virulent or the vaccine viruses.

MATERIAL AND METHODS

Samples

From January 2008 to January 2011, we collected and analyzed 249 field samples of tracheal swabs, tracheal tissues, lungs, and synovial fluids obtained from six turkey and one chicken farms (Table 1). All samples originated from different age categories of animals with various forms of respiratory and mobility problems.

The following vaccination programme was in force on the nursery farm:

1 day: Aviffa – aMPV by spraying and 21 days in the drinking watter

10 days: autogenous vaccine (ORT, *E. coli, Erysipelothrix rhusiopatiae, Pasteurella multocida*)

4–6 week: BYCOX (coccidiocidal)

35 days: Dindoral (HETV)

Treatment with amoxigal, (doxycyklin), enrofloxacin or tylan (sporadically) was administered in the first six weeks of life in the animals.

Vaccination on the fattening farms was not practiced, and only therapeutic and not antibacterial medication was administered.

Control strains

As positive controls, the following type strains were used:

Ornithobacterium rhinotracheale: CCM 4687 (Collection of microorganisms, Brno)

Mycoplasma synoviae: strain WVU 1853, type strain from 1991 (Freundt, Denmark)

Mycoplasma gallisepticum: NCTC 10115 (Bioveta, Ivanovice na Hane, Czech Republic)

Positive control for aMPV – Vaccine strain AVIFA-RTI (Merial, France) type B

Positive control ILT-TK strain (Bioveta, Ivanovice na Hane, Czech Republic)

PCR identification of *Ornithobacterium rhinotracheale* (ORT) and typing of strains using the M13 fingerprinting method

The extraction of bacterial DNA was performed directly from tissue samples using Nucleo spin blood extraction kit for the isolation of DNA from tracheal smears and Nucleo spin tissue from lungs (Machery Nagel, France) according to the manufacturer's instruction. Template DNA was amplified using primers with the following sequences: OR16S-F1 (5'-GAGAATTAATTTACGGATTAAG-3') and OR 16S-R1 (5'-TTCGCTTGGTCTCCGAAGAT-3') (Van Empel and Hafez 1999). For the preparation of the reaction mixture (25 μl) we used Combi PPP Master Mix (Top-Bio Praha, Czech Republic). Amplification conditions were: initial denaturation (5 min/94 °C), followed by 45 cycles of denaturation 30s/94 °C, annealing 1 min/52 °C and extension 1.5 min/72 °C. The reaction was terminated by final extension 7 min/72 °C (Van Empel et al. 1999). The expected length of the resulting PCR product was 784 bp.

For the detection of *O. rhinotracheale* lung samples and tracheal swabs were cultivated on meat-peptone blood agar supplemented with 10 μg/ml of gentamicine in microaerophilic environment for two days at 37 °C. Suspected positive colonies were examined and identified using the PCR method.

Typing of ORT isolates

Analysis using the M13 fingerprinting method (Thachil et al. 2007) was performed on six field

Table 1. Characteristics of the selected turkey and chicken farms used in this study

Farm	Type	Flock size	Age of animals/flock	Number of samples/flock
A	nursery	25 000	5–35days	22
B	fattening	3 × 24 000	5–20 week	83
C	fattening	3 × 6000	14 week	28
D	fattening	3 ×5500	14–21 week	40
E	fattening	3 × 6000	10–15 week	43
F	fattening	3 × 4500	8–17 week	30
G	poultry house	200 000	laying hen	3

isolates of *O. rhinotracheale*. Type strain ORT CCM 4687 serotype A was used as the control strain. Bacterial DNA was amplified using the M13 phage primer with the following sequence: 5'-TTATGTAAAACGACGGCCAGT-3'. For the preparation of the reaction mixture (25 µl) we used Combi PPP Master Mix (Top-Bio Prague, Czech Republic). For DNA extraction by boiling we used several colonies that were resuspended in 300 µl PBS. After centrifuging (14 000g, 3 min), the sediment was diluted in 200 µl of sterile deionized water and the suspension was boiled for 10 minutes. The material was subsequently diluted 1 : 1 in sterile deionized water. Amplification conditions were as follows: Initial denaturation (10 min/95 °C), followed by 35 cycles of denaturation 45 s/94 °C, annealing 3 min/40 °C and extension 4 min/72 °C. The reaction was terminated by a final extension of 10 min/72 °C. For separation and identification of DNA fragments (fingerprinting) we used electrophoresis in 2 % agarose gel with 0.5 µg/ml of ethidium bromide (Thachil et al. 2007).

Identification of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* using the nested-PCR method

Extraction of bacterial DNA was performed directly from the samples of tracheal smears using the Nucleo spin blood extraction and Nucleo spin tissue kits (Macherey Nagel) from lungs samples according to the manufacturer's instructions.

The region of 16S rRNA (Garcia et al. 1995) was amplified in *M. gallisepticum* and *M. synoviae* using primers with the following sequence for *M. synoviae*:
forward primer:
    5'-CGAAGGCAGCTAACTGG-3',
reverse primer:
    5'-TGCTTCTCTTTGTATCGTCC-3'
and for second round of amplification (nested PCR):
forward primer:
    5'-ACTAGTTGATRAAAACCATCGACGC-3'
reverse primer:
    5'-TCGGGCAGTCTCCTTAGATAAAG-3'
Samples for *M. gallisepticum* were amplified using the following primers:
forward primer:
    5'-ATGCTGAGAGGTAGAATAACC-3'
reverse primer:
    5'-CCACCTTACGGATTTGC-3'

and for second round amplification (nested PCR):
forward primer:
    5'-GGCGAAGGCGAGGACTTGGG-3'
reverse primer:
    5'-GCACCGAAGTATTCGCTCCGACAC-3'
Amplification conditions were identical for both mycoplasma species and the protocol included: 4 min initial denaturation at 94 °C, followed by 30 cycles of 35 s denaturation at 94 °C, 25 s annealing at 49 °C, and 90 s extension at 72 °C. The final extension took place for 10 min at 72 °C. The expected size of the amplified PCR fragment was 130 bp for *M. synoviae* and 312 bp for *M. gallisepticum*.

Identification of avian metapneumovirus using the RT-PCR method

Extraction of viral RNA was carried out using the Macherey Nagel extraction kit according to the manufacturer's instructions. The extracted RNA was subsequently frozen at –80 °C. Amplification of genes from the extracted RNA encoding nucleocapsid proteins was done using primers with the following sequence: forward primer: 5'-AGCAGGATGGAGAGCCTCTTTG-3', reverse primer: 5'-CATGGCCCAACATTATGTT-3' (Bayon-Auboyer and Jestin 1999). Both types (A and B) of aMPV are detected using these primers.

Amplification was done in a thermal cycler PTC-200 (MJ Research, USA) under the following conditions: reverse transcription 30 min/50 °C, initial denaturation 15 min/94 °C, amplification for 30 cycles (20 s/94 °C, 45 s/51 °C, 45 s/72 °C, final extension for 10 min at 72 °C. The PCR product was visualized using a trans-illuminator after 20 min of separation in a 2% agarose gel, stained with ethidium bromide. The expected size of the PCR product was 115 bp and it was compared with the DNA marker.

Identification of infectious laryngotracheitis virus using nested PCR method

Extraction of viral DNA was carried out using the Nucleo spin blood extraction kit and Nucleo spin tissue extraction kit (Macherey Nagel) according to the manufacturer's instructions. The extracted DNA was subsequently stored at –80 °C. Two sets of primers targeting the conserved regions of the glycoprotein E (gE) gene of ILTV were used (Chacon

Table 2. Detection of *Ornithobacterium rhinotracheale* by PCR

Farm	Number of samples	Positive/% positive
A	15	7/46.6
B	38	15/39.0
C	7	3/42.8
D	18	6/33.3
E	22	6/27.2
F	18	3/16.6

and Ferreira 2008). The primer sequences were: GE1S: 5'-CGTACTACCATCCTACAGACGGCA-3', GE2AS: 5'-CGTACAATGGTTCGGTCTTGGA-3' for amplification and GE3S: 5'-AGTCCTCTATA GCCATCCCCA-3' and GE4AS: 5'-CACCCCCG CGACGACGAAGT-3' for reamplification. The PCR protocol involved an initial denaturation step of 3 min at 94 °C, followed by 45 cycles of denaturating at 94 °C for 1min, annealing at 58 °C for 30 s and extension at 72 °C for 45 s, and final extension step at 72 °C for 10 min. Second-round amplification (nested PCR) was carried out using the same conditions. Samples that gave a product band at 219 bp after separation in 1% agarose gel were considered positive (Chacon and Ferreira 2008).

The specifity of the tests in ORT, MG, MS, aMPV aILT were checked using positive (type strains) and negative (type strains of other bacterial and viral pathogens) control strains in each examination. We
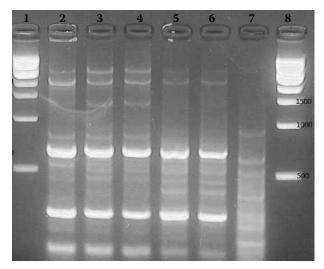

Figure 1. Application of M13 fingerprinting method to differentiate *Ornithobacterium rhinotracheale* isolates. Lane 1: 1 kb DNA ladder (New England Bio Labs); lane 2: type strain *O. rhinotracheale* (CCM 4687), lanes 3–6: field isolates ORT, lane 7: negative control, lane 8: 1kb DNA ladder

Table 3.Summary of samples tested by PCR for the detection of *Ornithobacterium rhinotracheale*

Type of samples	Number of samples	Positive/%positive
Tracheal smears	86	24/27.9
Lungs	29	13/44.8
Synovial fluids	3	2/66.6

selected these controls based on publications in which the methods were introduced and the specifity and sensitivity of the tests were controled.

RESULTS

The results of the determination of *O. rhinotracheale* prevalence are shown in Table 2. The highest prevalence of bacterial DNA was detected in lung tissue (Table 3).

The data in Table 2 show the prevalence of *O. rhinotracheale* in animals with respiratory signs (tracheitis, fibrous pneumonia) in the hatchery and on five fattening farms.

Comparison of the *O. rhinotracheale* type strain with examined field isolates showed that all strains gave an identical fingerprint pattern and belong to the same serotype A (Figure 1).

The prevalence of *Mycoplasma synoviae* is shown in Table 4. From the total number of 45 samples collected for detection of mycoplasma, tracheal smears (32 samples), lungs (11 samples), and synovial fluids (two samples) were all examined.

The only positive samples were detected in the synovial fluid of two animals on farm B. No examined sample was positive for *Mycoplasma gallisepticum*.

The prevalence of the etiological agents of *avian metapneumovirus* and infectious laryngotrachei-

Table 4. Detection of *Mycoplasma synoviae* by the nested PCR method

Farm	Tested samples	Positive/%positive
A	7	0
B	13	2/15
C	6	0
D	7	0
E	6	0
F	6	0

Table 5. Prevalence of aMPV and ILT as detected by PCR

Farm	aMPV		ILT	
	tested samples	positive/%positive	tested samples	positive/%positive
B	18	0	14	0
C	8	2/25	7	0
D	4	0	11	0
E	10	0	5	2/40.0
F	3	0	3	0
G	–	–	3	2/66.6

tis is shown in Table 5. From the total number of 43 samples (40 tracheal smears, three lung tissues) only two samples positive for aMPV were detected, on fattening farm C. A similar situation was found for infectious laryngotracheits virus: from the total amount of 43 samples (39 tracheal smears, four lung tissue) tested only four lung samples from fattening farm E and from poultry house G were found to be positive. All the tracheal smears were negative.

DISCUSSION

The application of molecular biological methods represents important progress in the laboratory diagnostics of turkey infections of both bacterial and viral etiology. These methods allow sensitive and specific diagnosis of infectious agents whose conventional isolation and identification is difficult and often unsuccessful.

While the detection of mycoplasmas and viral agents of respiratory diseases of turkey in our survey was rather sporadic, *O. rhinotracheale* was isolated in all six tested farms, mainly from samples of tracheal swabs and from the tissues of affected animals. Due to frequent contamination by other pathogens (*E. coli, Proteus* spp., *Pseudomonas* spp.), the isolation of *O. rhinotracheale* using a conventional cultivation procedure was successful in only a very small percentage of samples. One of the advantages of the PCR approach is that the strain does not have to be isolated and bacterial DNA can be detected directly in the extracted DNA from the examined material. The same conclusion was drawn by Ozbey et al. (2004).

In our diagnostic laboratory, we adopted a PCR test based on primers specific for the amplification of 16S RNA (van Empel and Hafez 1999). Using this method we have successfully detected

O. rhinotracheale in tracheal swabs and lung tissue from animals suffering from respiratory problems. In several cases we isolated *O. rhinotracheale* also from the synovial fluid of the ankle joint. The highest number of positive samples was obtained from lung tissue (44.8%).

The higher detection rate of the agent using PCR, versus the one obtained using conventional cultivation, indicates unambiguously that the PCR test is to be preferred in the diagnosis of *O. rhinotracheale*. It is generally known that due to the stability of DNA samples of tracheal swabs (which constituted the majority of our samples) do not have to be deposited in the transport medium, but can be transported to the laboratory in "dry" form.

For differentiation of *O. rhinotracheale* serotypes we introduced the M13 fingerprinting method. The most frequent turkey serotypes of ORT in European are A, B, D, as described by van Empel et al. (1997). Field isolates and the collection strain ORT were analysed and classified as the same serotype A. The same method was used by Tchachil at al. (2007) and was described as easy to use for diagnostic purposes.

The laboratory diagnostics of the pathogenic agent of mycoplasma disease of turkeys have recently been carried out using molecular biology methods. PCR and its various modifications (PCR-RFLP) are important because of the higher capture rate of the bacteria in clinical section material and also due to its difficulty of cultivation (Garcia et al. 1995). Such cultivation is possible only on chicken-embryonated eggs or on selective broths, forming characteristic colonies in the shape of "fried eggs". In summary, for the diagnosis of both types of mycoplasma the duplex PCR method, which targets the gene encoding the hemagglutinin protein could become a future alternative both for cultivation and for conventional PCR (Mardassi et al. 2005). The

aim for the future is to introduce this method into routine diagnosis of Mycoplasmas.

Although disease caused by *Mycoplasma gallisepticum* is spread worldwide and has a high prevalence in some less developed countries (Egypt, 40–60%; Bangladesh, 50–60%), we did not observe it in the flocks which we have tested. The explanation of this finding lies in preventive medication with antibiotics (tylan, enrofloxacin) in the first six weeks of life of the turkeys and adequate hygienic conditions on the farms.

The success of aMPV virus diagnostics depends on timely sampling. Replication of pneumovirus occurs in the upper respiratory tract and the virus is detectable only briefly (within 10 days after infection; Van de Zande et al. 1999). This can be one of the reasons for the low detection of viral RNA in all examined farms in our survey. Another reason for the low prevalence of aMPV is regular vaccination of turkey poults in the first day after hatching by spraying with the live vaccine Poulvac or by addition of the live vaccine AVIFA into drinking water every 7th and 28th day.

The most promising method we use in the diagnostic laboratory is RT-PCR for the detection of the gene encoding the nucleocapsid protein of the virus (Bayon-Auboyer and Jestin 1999). Using specific primers the method appears to be suitable for direct detection as well as for typing of viral RNA in field samples from turkeys. This method was compared with conventional virus isolation and was found to be three times more sensitive (Bayon-Auboyer and Jestin 1999).

The results of the diagnosis of the pathogenic agent of infectious laryngotracheitis in turkeys were similar; only four samples of lung tissue and pleura were positive from a total of 43 examined samples. Although poultry and pheasants are the natural and main carriers of virus, cases of latent ILT infection have been frequently described only in turkeys in Brazil (Portz et al. 2008). From a total number of 43 samples, we demonstrated the sporadic occurrence of ILT in the respiratory organs of turkeys from commercial farms using the nested PCR method. For the detection of ILT virus, the nested PCR method and virus isolation on chicken fibroblasts are more sensitive than isolation in chicken embryos (Portz et al. 2008). Abbas et al. (1996) proved that the PCR method is less sensitive than virus isolation, but more sensitive than histopathologic examination. Virus isolation requires live virus to be present, while the PCR method requires only the presence of viral DNA. Therefore, nested PCR appears to be a direct and sensitive method for the detection of the ILT virus (Portz et al. 2008).

In summary, this article describes a survey of the most frequent turkey respiratory pathogens occuring in flocks in the Czech Republic. The incidence of *O. rhinotracheale* was higher compared to other selected respiratory pathogens. We have applied molecular detection methods and have demonstrated their advantages in the detection of difficult-to-cultivate pathogens. Further studies are needed to monitor the incidence of these pathogens, to perform serotyping of isolates and to implement serological tests in diagnostics. Our future goal will be an evaluation of serological tests based on recombinant proteins as antigens which are currently being developed in our laboratory.

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