

Tissue Tropism of *Ornithobacterium rhinotracheale* in Chickens Determined by Culture and Nucleic Acid Detection

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Abstract

The aim of the present study was to evaluate the ability of *Ornithobacterium rhinotracheale* (ORT) to colonize chosen organs of chicks infected intratracheally (group A1), or intravenously (group A2), with the use of bacteriological methods and PCR. The bacteriological methods enabled to reisolate ORT bacteria from trachea and lungs of the birds from group A1 only on day 3 and 6 after infection. The PCR technique additionally detected the bacterial genetic material in these organs on the 9th day after infection, and gave positive results in the samples from air sacs until the 6th day of the experiment. In birds infected intravenously (A2) ORT was reisolated from liver on day 3 and from spleen on day 3 and 6 after infection, whereas the reisolation from the tibiotarsal joint occurred during the entire experimental period. PCR enabled to detect the bacterial DNA in the liver, spleen and lungs of chickens until the 9th day after infection and in case of tibiotarsal joint during the whole time of the study.

Key words: *Ornithobacterium rhinotracheale*, one day old chickens, experimental infection, tissue distribution, PCR

Respiratory tract infections constitute a significant problem in intensive poultry production. Studies conducted in France in 2005 showed that these infections caused 22% of the diagnosed diseases in the examined turkey flocks. Ornithobacteriosis is a relatively recently described bacterial disease of the respiratory tract caused by *Ornithobacterium rhinotracheale* (ORT) – a gram-negative, polymorphic, facultative anaerobic, non-motile and non-sporeforming bacteria (van Beek *et al.*, 1994). In the 1980s the bacterium was named “Pasteurella-like, pleomorphic gram-negative rod”, *Kingella* sp., or Taxon 28. Only in 1994 detailed phenotypic and genotypic analyses resulted in naming the bacterium *Ornithobacterium rhinotracheale* and presently it is classified into the *Flavobacteriaceae* family (Vandamme *et al.*, 1994). This microorganism was isolated for the first time in 1981 in Germany from the respiratory tract of turkey. Clinical cases of ornithobacteriosis were subsequently reported in ducks in Hungary in 1987, turkeys in Germany in 1991, and broiler chickens in Republic of South Africa (Hafez and Sting, 1999; van Beek *et al.*, 1994; Varga *et al.*, 2001). *O. rhinotracheale* were isolated from many bird species, such as: pheasant,

pigeon, rook, duck, ostrich, goose, guinea fowl, turkey, chicken, red-legged partridge, falcon (Amonsin *et al.*, 1997; Charlton *et al.*, 1993; Deverise *et al.*, 1995; Hafez and Lierz, 2010; Moreno *et al.*, 2009; Tsai and Huang, 2006; Vandamme *et al.*, 1994; van Empel and Hafez, 1999). The presence of the bacteria was predominantly detected in chickens and turkeys with clinical symptoms of pneumonia and airsacculitis. These species are the most sensitive to the infections caused by the pathogen (Marien *et al.*, 2006; Odor *et al.*, 1997; van Beek *et al.*, 1994; van Empel and Hafez, 1999). From 18 serotypes (A-R), serotype A is predominant among the chicken-isolates (96%) and the most frequent (54%) among the turkey isolates, which are more heterogeneously divided (A-E). There is no explanation for these differences in distribution but it has been shown that serotype A and C strains from chickens and serotype B, D and E strains from turkeys have a similar virulence for both chickens and turkeys (van den Bosh, 2001; Hafez, 2002). The presence of ORT has been reported in birds from many countries worldwide: England, Egypt, France, Israel, Iran, Canada, Mexico, Taiwan, Turkey and USA (Charlton *et al.*, 1993; Hung and Alvorado, 2001;

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Joubert *et al.*, 1999; Leroy-Setrin *et al.*, 1998; Tsai and Huang, 2006; Wyffels and Hommez, 1990). In Poland the clinical cases of ornithobacteriosis were confirmed for the first time in 1995 in turkeys (Ramza, 1996), and in 1998 in broiler chickens (Minta *et al.*, 1999). Serological examinations conducted in many countries have shown a significant degree of infection with ORT in poultry breeding farms, and broilers (Bock *et al.*, 1997; Canal *et al.*, 2003; Chansiripornchai *et al.*, 2007; Hafez and Sting, 1999; Odor *et al.*, 1997). Despite the fact that the etiological factor of ornithobacteriosis was discovered and classified several years ago the disease is still prevalent, causing significant economic losses in the poultry industry. The treatment of ORT in poultry flocks is difficult because of the improper diagnosis of many cases of the infection, and possible low effectiveness of the antibiotic therapy. The development of new diagnostic methods and effective immunoprophylaxis can contribute to limitation of the disease incidence. The aim of the present study was to evaluate the ability of *Ornithobacterium rhinotracheale* to colonize chosen organs of chickens after intratracheal, or intravenous infection, using bacteriological methods and PCR.

In this study a reference *Ornithobacterium rhinotracheale* strain – LMG 11343 (Catalog no ATCC 51464, LGC Promochem, UK) was used, which belongs to serotype A, and was isolated from the air sacs of chickens in Belgium. Bacteria were grown on Columbia agar for 48 h at 37°C under microaerophilic conditions. In the experiment 90 one day old chicks were used, which were purchased as embryonated SPF eggs (VALO, Lohmann-Tierzucht, Cuxhaven, Germany), incubated until hatching in the Division of Avian Diseases, Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, at Warsaw University of Life Sciences. The chickens were randomly divided into 3 groups (30 birds each): two experimental groups of infected animals (A1, A2) and one control group (A3). The birds were kept in laboratory coops and fed standard feed with water given *ad libitum*. Suspension of the ORT in sterile PBS was administered to the one day old chicks: birds from group A1 received intratracheally a dose of $2,34 \times 10^9$ CFU in 10 µl volume, group A2 was intravenously injected the same dose in 0,1 ml volume. The control group (A3) consisted of chicks that received only sterile PBS. The experiment was carried out for 15 days. Clinical and post-mortem examination of the birds from each group were done, and bacteriological analyses and PCRs were performed on collected samples. The samples for specific types of analyses were taken from 6 birds of each group every third day of the experiment, starting from the moment of infection, which gave a total of 5 collections. During the post-mortem examination samples of liver, spleen,

lungs, air sacs, trachea, and swabs from tibiotarsal joints were collected for further bacteriological analyses and PCR. The isolates from organ sections and tibiotarsal joint swabs were inoculated onto blood agar and blood agar with gentamicin (5 µg/ml) according to the protocol of Van Empel *et al.* (van Empel *et al.*, 1996). The plates were incubated at 37°C under microaerobic conditions for 48 h. The obtained colonies were identified by microscopic evaluation (Gram's stain reaction) and PCR technique (van Empel and Hafez, 1999). Extraction of total DNA from the organs' sections and tibiotarsal joint swabs was performed using a commercial "Sherlock AX" isolation kit (A&A Biotechnology, Poland) according to the protocol provided by the producer. The PCR technique was used to amplify a DNA fragment of *Ornithobacterium rhinotracheale* 16s rRNA gene. The amplification was performed using a pair of specific primers with the following sequences: OR16S-F1: 5'-GAGAATTAATTTACGGATTAAG-3' and OR16S-R1: 5'-TTCGCTTGGTCTCCGAAGAT-3' (van Empel and Hafez, 1999). The concentrations of PCR reagents in the reaction mixture were determined experimentally (Żbikowski *et al.*, 2006), while the temperature profile of the reaction was based on the previously published report (van Empel and Hafez, 1999). The PCR reaction mixture contained: 5,0 µl 10x Taq buffer (100 mM Tris HCl pH=8.8, 500 mM KCl) (MBI Fermentas, Lithuania), 5,0 µl dNTPs Mix (MBI Fermentas, Lithuania), 4,0 µl MgCl₂ (25 mM; MBI Fermentas, Lithuania); 25 pmol of each primer (IBB PAN, Poland); 1 or 5 µl of template DNA, 1 U Taq DNA polymerase (MBI Fermentas, Lithuania); and deionised water (Polpharma S.A., Poland) added to the final volume of 50 µl. The amplification comprised the following steps: initial denaturation at 94°C, for 5 min, 45 cycles consisting of DNA denaturation at 94°C for 30 s, primer annealing at 52°C for 60 s, and extension at 72°C for 90 s. The final extension was conducted at 72°C for 7 min (van Empel and Hafez, 1999). Amplification products were analysed by electrophoresis on 1% agarose gel with addition of 0.5 µg/ml ethidium bromide, conducted in TAE buffer, and constant voltage of 100 V. The expected size of the PCR product was 784 bp (Hung and Alvarado, 2001; van Empel and Hafez, 1999). For evaluation of the product size GeneRuler™ 100 bp DNA Ladder Plus (MBI Fermentas, Lithuania) was used as a reference. The study with the experimental infection of chickens with *Ornithobacterium rhinotracheale* was financed by the State Committee for Scientific Research in the years 2006–2008, as a part of scientific grant no: 3969/P01/2006/31, and was conducted with permission no: 4/2006 of the III Local Ethics Committee on Animal Experiments at Warsaw University of Life Sciences (SGGW).

Table I
Average body mass (g) of birds from experimental groups (A).

Group	Age of birds (days of life)					
	1	3	6	9	12	15
A1	36,57 ± 2,87	48,45 ± 3,50	68,23 ± 6,43	91,78 ± 7,19	112,50 ± 8,10	133,25 ± 8,77
A2	35,60 ± 1,98	36,27 ± 4,70 ^b	37,87 ± 5,80 ^b	41,81 ± 9,81 ^b	50,13 ± 15,74 ^a	68,74 ± 21,26 ^c
A3	36,14 ± 2,63	51,32 ± 4,91	71,99 ± 5,68	96,67 ± 12,83	121,48 ± 3,54	146,85 ± 5,26

* – statistically significant differences between the infected and control groups, at the level of significance $p \leq 0.05$

^a – statistically significant differences between the infected groups A1 and A2, at the level of significance $p \leq 0.05$

^b – statistically significant differences between the infected groups A1 and A2, at the level of significance $p \leq 0.01$

During the entire experimental period no typical signs of clinical ornithobacteriosis were observed in any of the infected birds. All chicks infected intravenously (group A2) were dejected, with improper feathering (prolonged presence of nestling feathers), and development retardation. The birds showed poor movement and decreased use of feed and water. In this group two chickens died during the observation period. Significant differences were noted with respect to the average body mass of birds (Table I). The post-mortem examination of the chickens injected intratracheally (A1) did not reveal any changes in the air sacs. On the 3rd, and 6th day after infection a small amount of exudation in the tracheal lumen was observed, together with lung changes in the form of unilateral or bilateral congestion. In the group infected intravenously (A2) all chickens showed emaciation, and breast muscles atrophy. On the 9th day after infection an inflammatory exudation was noted in tibiotarsal joints of the birds, and unilateral or bilateral lung congestion appeared on days 6 and 9 of the experiment. The control birds did not show any signs of changes in the examined organs.

Bacterial culture analysis revealed the presence of ORT bacteria in the samples from group A1, however the bacteria were reisolated from the trachea and lungs only on the 3rd and 6th day after infection. The PCR technique allowed for detection of the *O. rhinotrache-*

ale genetic material in these organs also on the 9th day, and additionally the bacterial DNA was amplified from the air sacs 6 days after infection. In the case of chicks infected intravenously (A2) ORT bacteria were reisolated from liver on day 3 after infection, and on days 3 and 6 from spleen, while in case of tibiotarsal joints isolation was possible during the whole time of the experiment. The PCR technique enabled to detect the bacterial DNA in the liver, spleen, and lungs until day 9 after infection, and the amplification of *O. rhinotracheale* genetic material from the tibiotarsal joints was possible in the collected samples from the entire experimental period. The presence of *O. rhinotracheale* was not confirmed with the use of both: bacteriological reisolation, and PCR in the samples of other organs of the infected birds (A1 and A2), and in any of the organs from the control chickens (group A3) (Table II).

The present study has shown that intratracheal infection of chickens with *O. rhinotracheale* did not induce specific clinical symptoms of ornithobacteriosis (results were similar to the control group), whereas the intravenous injection of ORT suspension caused intense unspecific symptoms, such as: dejection, reluctance to movement, reduced feed and water intake, decreased body weight, inhibition of development, and improper feathering. In chickens infected intratracheally the post-mortem examination revealed only

Table II
Results of bacteriological analysis and PCR of samples collected from the birds from groups A1, A2 and A3.

Days after infection		3			6			9			12			15								
Type of analysis		PCR			Bacterial culture			PCR			Bacterial culture			PCR			Bacterial culture					
Experimental group		A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Organs	liver	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	spleen	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
	trachea	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
	lung	+	+	-	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
	air sac	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	tibiotarsal joint	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-

+ positive result, - negative result

a small amount of exudation in the trachea and lungs congestion. In the case of the intravenous infections these symptoms were also observed in other organs. Additionally the intravenously infected birds were emaciated, had exudation from the tibiotarsal joints, and necrotic areas in the liver.

So far there have been very few detailed reports on the ability of ORT to colonize organs of experimentally infected chickens. In 2004 a study was published describing the experimental, intranasal and intraconjunctival infection of 3 weeks old turkeys with the LMG 9086 ORT strain (serotype A) in a dose of $8 \log_{10}$ CFU/bird (Marien *et al.*, 2006). The microorganism was isolated from the nasal mucous membrane, trachea and lungs until day 7 after infection, however no clinical symptoms of the disease were noted. The average number of colony forming units (CFU) of the reisolated strain per gram of the tracheal mucous membrane intensively increased, obtaining the maximal level on day 3–4 after infection (about $6 \log_{10}$ CFU/gram of tissue). After this time the values decreased rapidly. *O. rhinotracheale* could not be detected already on day 7 after infection (Marien *et al.*, 2006). ORT was also isolated from the ovary and oviduct 3 days after intramuscular infection of birds, and 14 days after intranasal and intravenous infection from the livers and oviducts of turkeys that did not show clinical symptoms of the disease. In this study *O. rhinotracheale* was not isolated from kidney and intestines. The presence of the bacteria in ovary and oviduct confirmed the transovarian infection, suggesting that the transmission of ORT occurs during acute phase of the disease (Travers, 1996).

The results of the present study confirmed that the *O. rhinotracheale* infection has a general nature, since the presence of bacteria was detected also in organs not belonging to the respiratory tract (liver, spleen, tibiotarsal joints). Using the inoculation method ORT could be reisolated only from trachea and lungs after the intratracheal infection, and from the liver, spleen, lungs, and tibiotarsal joints in case of the intravenous infection. The good adaptation of *O. rhinotracheale* to joints should be especially underlined. The PCR technique not only confirmed the affinity of the reisolated strain to the *O. rhinotracheale* species, but also allowed for detection of ORT genetic material in organs which gave negative results in the conventional bacterial culture, such as air sacs. The use of PCR enabled to obtain positive results in samples from the later days after infection, which remained negative in bacteriological analyses. The investigated ORT strain showed low virulence, and did not cause high mortality among birds. Available literature describes a high variability among ORT isolates in regard to mortality rate, however the exact mechanisms of this feature have not been established so far (Chansiripornchai, 2004).

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