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Etiopathogenesis and diagnostics of avian ornithobacteriosis

Etiopatogeneza i diagnostyka ornitobakteriozy ptaków

Summary. Avian ornithobacteriosis (ORT) is a disease entity associated with respiratory system, most often reported in chickens and turkeys. The etiologic agent of ORT is Gram-negative rod-shaped bacterium which after a detailed phenotypic and genotypic analysis performed by Vandamme was given the name *Ornithobacterium rhinotracheale* in 1994. This fact contributed to marked growth of *Ornithobacterium rhinotracheale* isolation and identification number worldwide. In Poland, the first isolations were recorded in 1995 from turkeys with respiratory disease symptoms. The optimal growth of these organisms is obtained when they are incubated on 5% sheep blood agar for at least 48h at 37°C under the micro-aerophilic conditions (5–10% CO_2). This disease may affect chickens and turkeys at any age, the birds manifest dyspnoea, sneezing, increased mortality rate and decreased egg production. Currently, 18 serotypes of ORT have been differentiated (from A to R). Owing to hardships in *Ornithobacterium rhinotracheale* isolation, efficient detection of the infected flocks is realized through serologic monitoring of ORT by the ELISA test. The direct diagnostics with PCR assays also proved extremely useful for identification purposes.

Key words: *Ornithobacterium rhinotracheale*, etiopathogenesis, diagnostics

INTRODUCTION

In 1991 Du Preez [van Beek *et al.* 1994] observed a new disease entity associated with the respiratory system in broiler chickens in South Africa and described it for the first time. The disease launched in the birds aged about 4 weeks with relatively mild respiratory signs including dyspnoea and sneezing. The symptoms were accompanied by increased mortality and worse performance. The disease persisted till the fattening period completion. At *post mortem* examination, pneumonia and foamy exudate (yoghurt-like) in abdominal air sacks were observed. The bacteriological examination performed by the

author revealed the slowly growing Gram-negative, pleomorphic, rod-shaped isolates that could not be classified as any of the known bacterial species.

As early as in 1987 similar rods were isolated in association with avian respiratory disease in Hungary, in 1990 in Belgium, whereas in the years 1991 and 1992 in Germany. Comparative studies on morphology and biochemical profile of these strains showed that they are identical with the strains isolated from birds in South Africa [van Empel *et al.* 1999a]. In 1993 some disease foci with similar respiratory signs were reported in Holland and again in Germany [Hafez *et al.* 1993]. In these flocks, there were also noted nasal and conjunctival sac discharge, sneezing and swelling of infraorbital sinuses associated with substantial decrease of body weight gains. Again, the bacteriological examination resulted in pleomorphic, Gram-negative rods isolation. Similar isolations were also performed from the respiratory diseased birds in Israel [Bock *et al.* 1995], in Belgium [Wyffels and Hommez 1990], in France [Leorat *et al.* 1994], in England [van Empel *et al.* 1999a] and in the USA [Charlton *et al.* 1993].

It is very much likely that *Ornithobacterium rhinotracheale* infections were earlier incorrectly diagnosed as viral diseases or bacterial infections induced by e.g. Pastereurella, Rimerella, Flavobacterium/Cytophaga spp. and NADH-dependent *Haemophilus paragallinarum* [Hafez *et al.* 1993; Mouahid *et al.* 1992, Bragg *et al.* 1997].

Initially, this newly isolated bacterium was designated as pleomorphic, Gram-negative rods [Charlton *et al.* 1993], Pasteurella-like [Hafez *et al.* 1993] or Kingella-like [van Beek *et al.* 1994]. It was only in 1994 when Vandamme and co-workers proposed for this new genus containing rRNA superfamily V the name Ornithobacterium [Vandamme *et al.* 1994].

The studies carried out by Vandamme *et al.* [1994] on the strains collected demonstrated that most probably the ORT strain had already been isolated from the turkey respiratory tracts as early as in 1981 as well as in 1983 from rooks. It was also confirmed that the strains isolated before 1990 in Belgium [Wyffels and Hommez 1990], in the USA [Charlton *et al.* 1993] and Israel [Bock *et al.* 1995] belonged to the *Ornithobacterium rhinotracheale* species. So far, no isolates from earlier than 1981 have been reported to be classed as *O. rhinotracheale* [Hinz and Hafez 1997].

Increased incidence and isolation of *O. rhinotracheale* from the clinical cases caused growing concern of the researchers this bacterium. Independent analyses made on the ORT strains collected from different countries allowed to classify bacteria on the grounds of their antigen structure. At present using the serotyping system described by Heddleston [Heddleston 1972], that is heat-stable antigen extraction in the agar gel precipitin test (AGP) and ELISA assay, the existence of up to 18 different serotypes of *Ornithobacterium rhinotracheale* (A-R) was indicated [van Empel *et al.* 1997]. The analysis of over one thousand strains obtained from chickens and turkeys [van Empel *et al.* 1997, van Veen *et al.* 2000] revealed that as much as 94% strains isolated from chickens and 57% from turkeys belong to the A serotype. The studies of van Empel et al. 1997 showed that all the strains of D and F serotype as well as most strains of serotype B (88%) and E (77%) came from the turkeys. Besides, the research results exhibited that the strains isolated from turkeys are more heterogenous and belong to seven serotypes, whereas those obtained from chickens appear to be more homogenous and most of them belong to the serotype A. On the basis of the obtained research results, the existence of some relationship was confirmed in the geographical distribution of individual serotype in the world.

All the strains from serotype C originated from California, while all the strains isolated in Great Britain and South Africa belonged to serotype A [van Empel 1997). Until recently, it has not been possible to state explicitly the reasons for the differences observed in the distribution of serotypes occurring in chickens and turkeys. In the serological examinations by the ELISA tests, stronger reactions were shown in the case of infections induced by the strains from serotype A compared to serotype B. The studies carried out by van Empel *et al.* [1997] on virulence of strains from different serologic types revealed a similar virulence level of the strains belonging to serotype A and B isolated from both, chickens and turkeys. A similar effect occurred between the strains from serotype C, D and E. These observations seem to exclude any specificity of ORT strains towards various bird species. Some differences in pathogenicity and severity of clinical sings may result from variable environmental factors and nutrition regimes over the rearing period.

Ornithobacterium rhinotracheale was isolated from the samples collected from chickens, turkeys, partridges, guinea fowl, gulls, pheasants, pigeons, rooks, quails, ducks, ostriches, geese [Charlton *et al.* 1993, Vandamme *et al.* 1994, van Empel and Hafez 1999b]. This disease may affect birds at any age, yet the older birds are more susceptible to it. In all these species, especially commercial poultry, *Ornithobacterium rhinotracheale* is capable of inducing this acute and highly contagious disease [Ryll 1996, Sprenger 1998, van Veen 2000]. However, the severity of clinical signs, duration of the disease and mortality are variable. They are influenced by numerous factors, mainly environmental ones, like poor management, inadequate ventilation, high stocking density, poor hygiene, high ammonia level, other diseases as well as secondary infections.

As for the diseases of complex etiology, *Ornithobacterium rhinotracheale* infections are accompanied by other respiratory cofactors, among others *Escherichia coli* [Sakai 2000], *Bordetella avium* [De Rosa 1997], viruses chiefly ND [Trawers 1996], IB [Erbeck 1998], TRT [Hafez 1998] and mycoplasmas mainly *M. synoviae* [Zorman-Rojs 2000].

In most researches with only ORT-challenged birds, the minimal lesions were shown in chickens and turkeys. Most frequently they were manifested by growth retardation without any noticeable clinical signs of the disease or capability of airsacculitis development [Back 1997, De Rosa 1997, van Empel 1997, 1999b, van Veen 2000]. In the case of challenge at the presence of other bacterial or viral pathogens, the lesions like airsacculitis, tracheitis or pneumonia were extremely marked. On the basis of these changes, van Empel [1999] showed a possibility of early isolation of ORT from the ciliated epithelium on the respiratory side of the air sacs as early as on the first two days post ORT challenge. The subsequent changes included opacification, edema and acute granulomatous airsacculitis. Generally, only the part of the bronchial associated lymphoid tissue (BALT) in the lungs became infiltrated and necrotic. Airsacculitis and pneumonia induced after aerosol ORT challenge of ND primed birds were fully established on 5–7 days post ORT infection and disappeared after 2 up to 3 weeks. The aerosol infection with no viral priming did not develop any pathological changes in this area, yet it produced a serological reaction comparable to that at the challenge with a viral priming. In the case of experimental challenge of flocks of meat turkey breeder birds aged 55 weeks, *Ornithobacterium rhinotracheale* was reported to survive in the ovary and oviduct without any noticeable clinical signs of the disease [Back 1998, Nagaraja 1998]. In other studies, the hatched broiler chicks and turkeys from the seropositive breeder flocks with

recognized ORT infection that were maintained in total isolation throughout the experimental period and provided with sterile feed and water, showed positive to ORT after aerosol exposure to ND and TRT virus. Then there was noted successful isolation of *O. rhinotracheale* from the air sacs undergoing the inflammatory process [van Empel 1997]. That implies that ORT may be transmitted in two ways, i.e. trans-ovarially or by the cloacal contamination. *Ornithobacterium rhinotracheale* may be also isolated from eggshells, yolk sacks of one-day-old chicks [van Empel 1997]. The research results combined with the field observations show that vertical transmission is also possible [Tanyi 1995]. To some extent, it explains the speed and range of ORT spread in the poultry flocks.

ISOLATION AND CLASSIFICATION OF ORT

Ornithobacterium rhinotracheale is a Gram-negative, non-motile, nonsporulating, pleomorphic rod [Vandamme *et al.* 1994]. So far, no specific structures like cilia, fimbriae, plasmids or properties specific for toxic activity have been found within this bacterial species [Leroy-Setrin 1998].

The organisms grow readily on blood agar with 5% sheep blood being incubated for at least 48h under micro-aerophilic conditions (5–10% CO_2). In these conditions, the first *Ornithobacterium rhinotracheale* colonies develop as early as after 24 h; however, after 48h-culture typical small grey to grey-white colonies were observed, sometimes with reddish glow and always with a clear butyric acid-like odor. In the primary isolation, most of the ORT isolates show marked differences in the colony size, i.e. 1–3 mm after 48h incubation period. Growth on the liquid media exhibits higher pleomorphism compared to the solid ones, a bacterial cell (of 0.2 up to 6 μm thickness) shows more substantial differences in length (0.6–5 μm) and can develop clumps composed of several thousands of single cells. Still, not all the strains grow so readily even on the nutrient-rich liquid media, such as Todd-Hewitt broth or Brain Heart Infusion broth (BHI) supplemented with serum. Up till now, no highly selective and differential culture medium has been available. That is why a frequent difficulty in *Ornithobacterium rhinotracheale* isolation on the standard media appears to be contamination of samples with concomitant microflora, like *E. coli* or *Proteus spp.* to inhibit their growth, it is recommended to use sheep blood agar medium that contains gentamicin and polymyxin (both 5 μg/ml) or gentamicin alone (10 μg/ml) [van Empel 1997]. Due to the fact that approximately 90% *Ornithobacterium rhinotracheale* strains are resistant to both antibiotics, sheep blood agar without antibiotic addition should always be included in the isolation procedures for the control purposes.

Quite a difficult technique of *Ornithobacterium rhinotracheale* isolation from the respiratory tract may be successfully improved with some changes in the routine isolation methods, i.e. longer incubation period in the conditions 5 up to 10% CO_2. This modification resulted in a considerably higher rate of the ORT isolation from the cases of airsacculitis and uncomplicated pneumonia reported in meat turkey breeds and broiler chickens.

Van Empel *et al.* [1997] performed the analysis of the research results of 56 strains originating from different sources and belonging to different serotypes. On these grounds, a biochemical profile of *Ornithobacterium rhinotracheale* has been established. All the strains proved to be positive for oxidase, urease, β-galactosidase, arginine dehydrolase, alkaline phosphatase, esterase lipase, leucine arylamidase, cystine arylamidase,

acid phosphatase, phosphohydrolase, α-glucosidase, N-acetyl-β-glucosaminidase and acid production (without gas) from glucose, fructose, lactose and galactose. On the other hand, the colonies were negative for nitrate reduction, production of catalase, lysine decarboxylase, ornithine decarboxylase, indole production, gelatinase, esterase, lipase, chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase as well as production of acid from maltose, sucrose, fructose and ribose. There was no growth on MacConkey agar and negative – motility test.

In the routine laboratory diagnostics, it seems more helpful to use the commercial biochemical test-kits. A great number of studies were carried out using the kit API 20NE [van Empel 1997, 1998]. However, the obtained test results may prove less stabile owing to the fact that not all the ORT strains grow on liquid media commonly used in the identification system. The use of the adapted media cause that the biochemical capabilities of *Ornithobacterium rhinotracheale* become more cohesive irrespective of the species or the serotype of strains.

Due to some deviations from the recommended procedures and the fact that *Ornithobacterium rhinotracheale* has not been included in the database of API resources, the routine laboratory diagnostics should include additional tests that would assure the explicit identification of a strain. For this purpose, API-20NE test (incubation at 30^{o}C temperature) was a most preferred method combined with agar gel precipitation test (AGP). About 1150 *Ornithobacterium rhinotracheale* strains were examined with the API-20NE test by van Empel *et al.* [1997, 1998] which showed that 99.5% of the strains gave a reaction code of 0-2-2-0-0-0-4 (61%) or 0-0-2-0-0-0-4 (38.5%), which being confirmed in the AGP test gave a really high identification scores.

Another commercial identification system used for ORT was RapID NF Plus system. However, the tests performed by Post *et al.* [1999] on 110 ORT strains showed a possibility of different identification codes, i.e. 4-7-2-2-6-4, 4-7-6-2-6-4, 6-7-2-2-6-4 like while using API-ZYM test procedure by Hafez in 1996, Chin and Droual in 1997 as well as Ryll and Hanz in 1997. All the tests provided solely a possibility to establish rather constant enzymatic characteristics of *Ornithobacterium rhinotracheale*. So far, this system has not been furnished with the data necessary for identification and, thus, it proves less useful in the routine laboratory diagnostics.

Because *Ornithobacterium rhinotracheale* is difficult to isolate, fast detection of the infected flocks is carried out through the serological monitoring using ELISA assays [van Empel and Hafez 1999b]. The antibodies presence may be detected in one-day-old chickens, in egg yolk as well as in the birds with clinical signs of the disease. Therefore, the ELISA test may be implicated to aid in diagnosis of ORT. The high serotype specificity of the ELISA test is a disadvantage in this case, but the commercial kit has been developed so that it could detect antibodies directed against 9 out of 18 serotypes recognized so far. The antibodies rise between 1 and 4 weeks post infection and then rapidly fall. Under the conditions of experimental aerosol challenge with ORT, the antibodies were detected as early as 5 days after induced infection [van Empel 1998]. The level of antibodies measured by the ELISA test after the experimental challenge appeared to be considerably higher than that following a natural infection, and it persisted longer. It may be a result of more intensive immunization of birds under the experimental conditions.

Extremely useful for identification proved to be the direct diagnostics by a polymerase chain reaction assay (PCR). The primers OR16S-F1 (5`-GAGAATTAATTTACGG-

ATTAAG) and OR16S-R1 (5`-TTCGCTTGGTCTCCGAAGAT) described by van Empel [1999a] are highly specific only for a 784bp fragment on the 16SrRNA gene of *Ornithobacterium rhinotracheale* and at the same time are not consistent with any other closely related bacteria occurring in a genome. Another possibility for the typing is using rep-PCR "fingerprints" with M13 (5`-TATGTAAAACGACGGCCAGT) and ERIC IR (5'-ATGTAAGCTCCTGGGGATTCAC) primers described by Hafez and Beyer in 1997. In this case, however, some discrepancies between the investigated serotypes were observed.

Recent years in Poland have been marked with an increasing interest in a rod-shaped bacterium ORT in the research centers. The serological tests have confirmed substantial spread of this pathogen. Wieliczko *et al.* [2001] showed the presence of antibodies against *Ornithobacterium rhinotracheale* in 20% turkey breeder flocks, 76% hen breeder flocks, 55% slaughter turkey flocks and 46.7% slaughter chicken flocks. However, in the year 2006, Szeleszczuk *et al*. studying the chicken broiler flocks, revealed the presence of antibodies against ORT in 36.6% of sera obtained from the day-old chicks, 8.46% of sera from the 3-week-old birds and 45.65% positive results in the sera collected from the birds at the late productive period.

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Streszczenie. Ornitobakterioza ptaków to jednostka chorobowa układu oddechowego najczęściej występująca u kur i indyków. Czynnikiem etiologicznym ornitobakteriozy jest Gram-ujemna pałeczka, którą w 1994 r. Vandamme po szczegółowej analizie fenotypowej i genotypowej nazwał *Ornithobacterium rhinotracheale*. Przyczyniło się to do znaczącego wzrostu ilości izolacji i identyfikacjilacji *Ornithobacterium rhinotracheale* na całym świecie. W Polsce pierwsze izolacje nastąpiły w 1995 r. od indyków z objawami ze strony układu oddechowego. Optymalny wzrost tych organizmów jest uzyskiwany poprzez inkubację na agarze z dodatkiem 5% krwi baraniej przez co najmniej 48 h w warunkach mikroaerofilnych (5–10% CO_2) w 37°C. Choroba może wystąpić u kur i indyków w każdym wieku. Objawia się dusznościami, kichaniem, zwiększoną śmiertelnością oraz gorszymi efektami produkcyjnymi. Do chwili obecnej wyróżniono 18 serotypów *Ornithobacterium rhinotracheale* (A do R). Ze względu na trudności w izolacji pałeczki, szybkie wykrywanie stad zakażonych prowadzi się poprzez monitoringowe badania serologiczne z wykorzystaniem testów ELISA. Niezmiernie użyteczna w identyfikacji okazała się również diagnostyka bezpośrednia techniką PCR.

Słowa kluczowe: *Ornithobacterium rhinotracheale*, etiopatogeneza, diagnostyka