

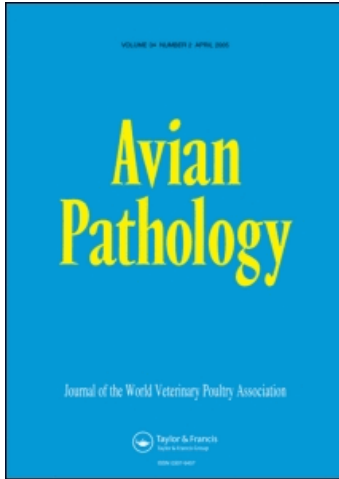
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Antibiotic sensitivity and resistance in *Ornithobacterium rhinotracheale* strains from Belgian broiler chickens

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Establishing the antibiotic sensitivity of the avian respiratory pathogen *Ornithobacterium rhinotracheale* is difficult because of the organism's complex growth requirements and the unusually frequent occurrence of resistance. The minimal inhibitory concentrations of 10 antibiotics were determined for 45 strains of *O. rhinotracheale* from Belgian broiler chickens collected from 45 farms between 1995 and 1998. They were compared with the type strain, which was isolated from a turkey, and a strain isolated from a rook. All the broiler strains were resistant to lincomycin and to the β -lactams ampicillin and ceftiofur. Less than 10% of the strains were sensitive to the macrolides tylosin and spiramycin, tilmicosin and flumequine. A few strains were sensitive to enrofloxacin and doxycycline. All strains were sensitive to tiamulin.

Introduction

Few data are available on the sensitivity and resistance to antibiotics of *Ornithobacterium rhinotracheale*, a cause of respiratory disease in chickens, turkeys and other gallinaceous birds (Vandamme *et al.*, 1994; van Empel & Hafez, 1999). This is probably due to the exacting growth requirements and fastidious nature of this bacterial species. The normal antibiotic sensitivity test media do not support growth of *O. rhinotracheale*. Furthermore, its slow growth causes abnormally large inhibition zones in the routinely used diffusion test antibiograms, which are difficult if not impossible to interpret. This necessitates the application of quantitative dilution test procedures to determine minimal inhibitory concentrations (m.i.c.) on rich blood-supplemented media.

Another and somewhat unusual cause of difficulties in the interpretation of *O. rhinotracheale* sensitivity tests concerns its extraordinarily high rates of acquired resistance against certain antibiotics (Devriese *et al.*, 1995). It is well known that each bacterial species has its own normal

sensitivity level against a given antibiotic as a result of cell wall composition, the presence or absence of certain receptors, etc. All strains belonging to the same species show similar m.i.c. values, usually situated within a narrow range of concentrations of this antibiotic, unless they have acquired resistance (Walker, 2000). These normal levels are well known with the common pathogenic bacterial species and the widely used antibiotics, and they are determined and published when a new antibiotic is introduced. On the other hand, when a new pathogen is detected, it is of importance to gain an insight to the normal sensitivity levels. This offers no difficulties except in cases where growth conditions are exacting and when resistance rates are high, as is the case with *O. rhinotracheale*. In an earlier preliminary study on a small number of strains (Devriese *et al.*, 1995), we used strains from rooks in order to detect the normal m.i.c. values. These strains, originally isolated in Northern Germany by K. H. Hinz, had been included in the species description of *O. rhinotracheale* by Vandamme *et al.* (1991)

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Table 1. Minimal inhibitory concentrations of antibiotics on 45 *Ornithobacterium rhinotracheale* strains from broilers and two reference strains

Antibiotic	Number of strains with m.i.c. ($\mu\text{g/ml}$) of									
	≤ 0.12	0.25	0.5	1	2	4	8	16	32	≥ 64
β-Lactams										
Ampicillin	R ^a		<i>I</i> ^a	9	9	<i>T</i> ^b + 16	9	1		
Ceftiofur	R			<i>T</i> + 1	3	3	17	17	4	
Macrolides and related antibiotics										
Tylosin	R + T	1		5		1	3	5	7	23
Spiramycin					R + T	1		1		43
Lincomycin	R + T									45
Tilmicosin	T		R + 2					1	1	41
Quinolones										
Flumequine	1	R + T + 2					3	4	28	7
Enrofloxacin	R + T + 3	2		11	29					
Tetracycline										
Doxycycline	2	R + T + 6	1		1	1	10	24		
Pleuromutilin										
Tiamulin	T + 35	R + 10								

^a Resistant strains in italics.

^b Reference *O. rhinotracheale* strains: T, type strain; R, strain from a rook.

The present article extends these observations to a wider range of strains and to certain additional antibiotics of possible therapeutic importance.

Materials and Methods

Antibiotics were obtained as standard powders with known activity: ampicillin, tylosin, lincomycin and doxycycline from Sigma (St. Louis, MO, USA), ceftiofur from Pharmacia & Upjohn (Puurs, Belgium), spiramycin from Merial (Brussels, Belgium), tilmicosin (Elanco, Nieuwegein, The Netherlands), flumequine from Sanofi (Brussels, Belgium), enrofloxacin from Bayer (Brussels, Belgium), and tiamulin from VMD (Arendonk, Belgium). Stock solutions containing 1000 or 10000 $\mu\text{g/ml}$ were prepared in phosphate buffer (pH 8) (ampicillin), ethanol (spiramycin, tylosin), distilled water with a minimal amount of NaOH (enrofloxacin and flumequine), or distilled water (remaining antibiotics). To obtain the desired final concentrations, intermediate dilutions containing 1000, 100 and 10 $\mu\text{g/ml}$ were prepared with distilled water. These dilutions were added to molten Columbia agar (Oxoid, Basingstoke, UK) in 50 ml amounts in bottles at 50°C and supplemented with 5% sheep blood. Three plates each containing 15 to 20 ml medium were prepared from one bottle. Antibiotic-free control plates were made using the same medium. Plates were dried for approximately 15 to 30 min on a laminar flow bench.

Forty-five *O. rhinotracheale* strains were isolated between 1995 and 1998 in as many different broiler farms from tracheal swabs on Columbia agar with 5% sheep blood with or without 5 $\mu\text{g/ml}$ polymyxin (Sigma) and 5 $\mu\text{g/ml}$ gentamicin (Sigma) after 2 days of incubation at 37°C in a 5% CO₂-enriched environment. The strains were identified by colony and microscopic morphology, and by API20NE galleries (BioMérieux, La Balme-les-Grottes, France). Their serotype (van Empel *et al.*, 1997) was determined in agar gel precipitation tests (courtesy of Dr P. C. M. van Empel, Intervet, Boxmeer, The Netherlands). All field strains were shown to belong to serotype A. The *O. rhinotracheale* type strain LMG 9086^T originally isolated from a turkey and strain LMG 11553 from a rook obtained from the LMG culture collection (Ghent University, Ghent, Belgium)

were included as reference strains. The antibiotic susceptibility reference strain *Staphylococcus aureus* ATCC 29213 was inoculated on each plate as a control for antibiotic content.

Preserved strains were grown on Columbia blood agar plates in 5% CO₂ in air. Purity was checked and representative colonies were inoculated in brain heart infusion (5 ml) tubes. Suspensions matching 0.5 McFarland were prepared in saline with a Vitek Systems ATB1550 photometer (BioMérieux, Marcy l'Etoile, France), and inoculated on the antibiotic and control plates using a Denley Multipoint Inoculator (Mast, London, UK). In this way, approximately 10⁵ colony forming units of each strain was inoculated on the plates.

Results were read after incubation at 37°C in 5% CO₂ for 2 days. The m.i.c. was recorded as the lowest concentration that completely or nearly completely inhibited growth, thus disregarding faint hazes of growth or single colonies. Strains were considered resistant when the m.i.c. values were three or more twofold dilution steps higher than those of the reference strains.

Results

The m.i.c. values obtained are presented in Table 1. None of the strains from domestic poultry, including the type strain (T) originating from a turkey, was fully susceptible to the β -lactam antibiotics ampicillin and ceftiofur. With the macrolides, lincomycin, the quinolones and doxycycline normal susceptibility levels were seen with the type strain and with one, two or a few strains from broilers. The m.i.c. values of tylosin with resistant strains were distributed over a wider range (1 to over 64 $\mu\text{g/ml}$) than those of the other macrolide antibiotics spiramycin and tilmicosin. All strains from broilers were highly resistant to lincomycin. All except two of the 40 strains resistant to flumequine

showed decreased sensitivity to enrofloxacin. Nine strains (20%) were fully sensitive to doxycycline, the others being 40- to 100-fold less susceptible. All strains were susceptible to tiamulin (m.i.c. value, 0.25 µg/ml or lower).

Discussion

The antibiotic treatment of respiratory disease in poultry has always been problematic. Many antibiotics cannot be used because of poor resorption from the gut or because of high cost. van Beek *et al.* (1994) reported that conventional oral therapy showed poor results in the treatment of *O. rhinotracheale*-infected turkeys, especially when pneumonia was present. These failures were probably due, at least in part, to the widespread occurrence of acquired resistance in this bacterium. Although m.i.c. values of resistant strains are apparently low with the β-lactams and the quinolones, and the term "decreased sensitivity" might be more appropriate to characterize their antibiotic sensitivity status, the therapeutic efficacy of these antibiotics on infections caused by such strains is questionable. This can be concluded from the comparisons of kinetic study results with m.i.c. data, on the one hand, and from experimental therapy studies of infections with bacterial strains of different sensitivities on the other (Brown, 1996; Ziv *et al.*, 1997; Tell *et al.*, 1998; Knoll *et al.*, 1999). However, the latter type of studies has never been performed with *O. rhinotracheale*.

Our results are likely to be representative of the current situation in *O. rhinotracheale* in broilers in Belgium. They confirm the earlier findings with strains from eight chicken farms except that the quinolone resistance rates were much higher in the present study. It is difficult to compare the present results with data from other investigators (reviewed by van Empel & Hafez, 1999) since their test methods and interpretative criteria have not, or only very briefly, been defined and the criteria for sensitivity and resistance may have differed. For example, it is possible that the ampicillin and enrofloxacin m.i.c. values regarded as indicating resistance in our study may have been classified in the susceptible category in other studies.

The m.i.c. determinations performed in the present study are not easy to incorporate in routine bacteriological examinations. Even ready-made broth dilution microtitre plates cannot be used without first checking of their suitability for tests with *O. rhinotracheale*. The E test procedure (AR biodisk, Solna, Sweden) may be more suitable, provided that strips containing the antibiotics of interest are available. It may be possible to interpret inhibition zone diameters of disk or tablet diffusion tests with *O. rhinotracheale* according to the criteria in use with certain fastidious bacteria of human medical importance (Doern, 1995). These have to be adequately controlled by the inclusion of

O. rhinotracheale strains with known sensitivity.

The resistance mechanisms active in *O. rhinotracheale* are unknown except in the case of the β-lactams, in which a β-lactamase has been demonstrated (Devriese *et al.*, 1995). This enzyme was only absent in the three normally sensitive strains studied at that time, all of which originated from rooks. The β-lactamase involved appears to affect a wide range of β-lactam antibiotics. In this study, penicillin G was also shown to be less active on strains carrying the enzyme, in a manner similar to the broad-spectrum penicillin ampicillin and the cephalosporin, ceftiofur. Because of the common occurrence of cross-resistance, it is probable that other members of these antibiotic groups are less active on the less sensitive strains, as are other macrolides, quinolones and tetracyclines on strains showing resistance to the representatives of these antibiotic families tested in the present article. The detection of β-lactamase activity in the strains with decreased sensitivity to ampicillin and ceftiofur confirms the interpretation of the m.i.c. values in terms of sensitivity and resistance, as indicated in Table 1. This finding is remarkable because none of the poultry strains, including the type strain isolated from a turkey, can be considered normally sensitive.

It can be concluded that the therapeutic potentials on *O. rhinotracheale* of all antibiotics tested are likely to be impaired by the widespread occurrence of resistance, except in the case of tiamulin. Whether or not this antimicrobial is suitable for the treatment of this type of infections remains to be determined.

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RÉSUMÉ

Sensibilité et résistance aux antibiotiques des souches d'*Ornithobacterium rhinotracheale* isolées de poulets de chair en Belgique

L'établissement de la sensibilité aux antibiotiques de l'agent pathogène respiratoire aviaire *Ornithobacterium rhinotracheale* est difficile du fait des exigences complexes de croissance de l'organisme et de l'inhabituelle fréquence d'apparition de résistance. Les concentrations minimales inhibitrices de 10 antibiotiques ont été déterminées pour 45 souches *O. tracheale* isolées de poulets de chair provenant de 45 élevages entre 1995 et 1998. Elles ont été comparées à celle de la souche type, qui a été isolée d'une dinde et d'une souche isolée d'un freux. Toutes les souches isolées de poulets de chair se sont avérées résistantes à la lincomycine et aux β lactamines, ampicilline et

ceftiofur. Moins de 10 % des souches se sont révélées sensibles aux macrolides tylosine et spiramycine, tilmicosine et flumequine. Quelques souches se sont avérées sensibles à l'enrofloxacin et à la doxycycline. Toutes les souches étaient sensibles à la tiamuline.

ZUSAMMENFASSUNG

Antibiotika-Empfindlichkeit und -Resistenz bei Stämmen von *Ornithobacterium rhinotracheale* aus belgischen Broilerküken

Das Feststellen der Antibiotika-Empfindlichkeit des Erregers aviärer Atemwegserkrankungen *Ornithobacterium rhinotracheale* ist wegen der komplexen Kultivierungsansprüche und des ungewöhnlich häufigen Vorkommens von Resistenz schwierig. Die minimalen Hemmkonzentrationen von 10 Antibiotika wurden für 45 *O. rhinotracheale*-Stämme bestimmt, die zwischen 1995 und 1998 aus belgischen Broilerküken isoliert wurden. Sie wurden mit dem Standardstamm verglichen, der aus einer Pute isoliert wurde, und mit einem aus einer Saatkrähe isolierten Stamm. Alle Broilerstämme waren gegen Lincomycin und die β -Laktame Ampicillin und Ceftiofur resistent. Weniger als 10% der Stämme waren gegen die Makrolide Tylosin und Spiramycin, Tilmicosin und Flumequin empfindlich. Ein paar Stämme waren gegen Enrofloxazin und Doxycyclin empfindlich. Alle Stämme waren Tiamulin-empfindlich.

RESUMEN

Sensibilidad y resistencia a antibióticos de cepas de *Ornithobacterium rhinotracheale* aisladas de pollos broiler belgas

Establecer la sensibilidad a antibióticos del patógeno aviar *Ornithobacterium rhinotracheale* es difícil debido a los complejos requerimientos de crecimiento y a la inusual frecuencia de aparición de resistencias. Se determinaron las concentraciones inhibitorias mínimas de 10 antibióticos para 45 cepas de *O. tracheale* aisladas de pollos broiler belgas recogidos de 45 granjas entre el 1995 y el 1998. Se compararon con la cepa tipo, que fue aislada de pavo, y una cepa aislada de grajos. Todas las cepas de broiler mostraron resistencia a la lincomicina y a los β -lactámicos, ampicilina y ceftiofur. Menos del 10% de las cepas eran sensibles a los macrólidos, tilosina y espiramicina, tilmicosina y flumequina. Algunas pocas cepas fueron sensibles a la enrofloxacina y a la doxiciclina. Todas las cepas fueron sensibles a la tiamulina.