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Characterization and identification of *Ornithobacterium rhinotracheale* strains

Diploma Thesis

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submitted by

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Abbreviations

°C	degree celsius
ELISA	Enzyme Linked Immunosorbent Assay
h	hours
µl	microliter(s)
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation – Time of Flight
MIC	minimal inhibitory concentration
MH	Müller-Hinton
PCR	Polymerase Chain Reaction
sp.	species
spp.	species pluralis

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1 Introduction and aim of the study

The bacteria *Ornithobacterium (O.) rhinotracheale* is a major pathogen of turkeys and other poultry, causing mortality, respiratory symptoms, growth suppression and moving disorders (Siegmann 2011; Swayne 2020; Barbosa et al. 2019). *O. rhinotracheale* infections are considered to be influenced by many factors such as coinfections, inadequate ventilation, poor hygiene and high stocking density and can have great economic impact (van Empel and Hafez 1999; van Veen et al. 2001; Swayne 2020).

Turkeys are most affected, but also broilers, turkey breeders and broiler breeders can become diseased (Swayne 2020). Data from various countries showed, that *O. rhinotracheale* is spread worldwide and effectivity of antibiotic treatment has declined over time (van Empel and Hafez 1999; van Veen et al. 2001; Swayne 2020; Szabó et al. 2015). The reason for this is the ability of *O. rhinotracheale* to acquire antimicrobial resistances against different classes of antibiotics (van Veen et al. 2001; Barbosa et al. 2019; Swayne 2020).

With protection through vaccination against *O. rhinotracheale* mostly described as not sufficient (Barbosa et al. 2019), an effective antibiotic treatment is often urgently needed. No international standard protocol for antibiotic susceptibility testing for *O. rhinotracheale* exists and many different methods have been used in research over the last two decades (van Veen et al. 2001; Malik et al. 2003; Szabó et al. 2015). There is plenty of literature on antibiotic susceptibility of *O. rhinotracheale* strains from various countries, but no data are available yet from Austria. The MICRONAUT-S system (MICRONAUT-S©, MERLIN Gesellschaft für mikrobiologische Diagnostika mbH, Germany) is a semi-automated microdilution method suitable for routine laboratory diagnostic and allows to follow CLSI guidelines for susceptibility testing (MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH; Pfennigwerth et al. 2019; Cordovana and Ambretti 2020; Baquer et al. 2021).

The aim of this thesis was to establish a protocol for susceptibility testing of *O. rhinotracheale* with MICRONAUT-S and to evaluate the antibiotic susceptibility of *O. rhinotracheale* strains isolated in Austria between 2002 and 2021.

2 Literature survey

2.1 Etiology and significance

Ornithobacterium rhinotracheale is a bacterium causing mainly respiratory symptoms, mortality and reduces growth in turkeys and broilers. It can also be associated with a drop in egg production in breeding flocks. The disease is present throughout the world and can cause high economic losses. (Swayne 2020; Siegmann 2011)

Ornithobacterium rhinotracheale belongs to the genus *Ornithobacterium*, which is a member of the *Flavobacteriaceae* (Swayne 2020). *Flavobacteriaceae* are a part of the superfamily V rRNA (Barbosa et al. 2019). Related bacteria with relevance for poultry are *Riemerella anatipestifer* and *Coenonia anatina* (Swayne 2020).

O. rhinotracheale is gram negative, non-motile and pleomorphic to rod-shaped. Its size varies from 0.2 µm x 0.6 µm to 0.9 µm x 5 µm. (Swayne 2020)

The bacterium grows on sheep-blood agar, tryptose soy agar and chocolate agar in 24h to 48h aerobically, microaerobically and anaerobically (Swayne 2020). Best growth occurs on blood agar in an microaerophilic or anaerobic environment at 37°C (Siegmann 2011). It does not grow on MacConkey agar (Swayne 2020). Growth in nutritious fluid media such as brain heart infusion broth or Pasteurella broth is also possible (Swayne 2020). *O. rhinotracheale* characteristically develops small, grey colonies with no hemolysis (Siegmann 2011). Also colonies with a reddish colour (Swayne 2020) and β-hemolysis are described (Zahra et al. 2013; Tabatabai et al. 2010).

Using different techniques, such as enzyme linked immunosorbent assay or agar gel precipitation, 18 serotypes (A to R) can be distinguished (Swayne 2020). Most isolates from broilers belong to serotype A, while isolates from turkeys distribute mostly to serotypes A, B and D (Siegmann 2011). Recent research showed that serotypes F, K and M form an own cluster in phylogenetic analysis which was backed up by results from whole genome sequencing (Alispahic et al. 2021). The three serotypes might even belong to a different species in the *Ornithobacterium* genus (Alispahic et al. 2021).

2.2 Epidemiology, clinical signs and pathology

Besides from farmed poultry the pathogen was found in a variety of wild birds, including birds of prey, pigeons and pheasants, which may serve as a reservoir. The transmission of the bacterium can be direct through contact with infected animals or indirect through living or dead vectors. Furthermore, transmission through aerosols is possible. The incubation time is between one and four days after experimental infection. (Siegmann 2011)

O. rhinotracheale causes different clinical symptoms, which can differ significantly in their severity, but the mortality and duration of the disease can vary considerably. Influencing factors are poor management, bad air quality, high stocking density, hygiene and other diseases. (Swayne 2020)

Disease due to *O. rhinotracheale* was first described in 1994 (Vandamme et al. 1994) and 1997 an outbreak in Austrian turkeys has been reported (Hafez and Friedrich 1998).

Turkeys can already be infected at an age of two weeks, although most infections and more severe symptoms occur at 14 weeks or older (Swayne 2020). Symptoms include apathy, decreased food intake and weight gains, nasal discharge, sneezing, dyspnoea and facial edema, as well as movement disorders due to arthritis (Siegmann 2011). Mortality varies from 1 % to 15 %, but can also rise up to 50 % (Swayne 2020).

Clinical signs in broilers may appear at three to six weeks of age and include apathy, decreased food intake and weight gains, nasal discharge, sneezing, dyspnoea and facial edema (Swayne 2020; Siegmann 2011). Mortality in broilers can be between 2 % and 10 %, but can go up to 20 % in rare cases (Swayne 2020).

Broiler breeders are mostly affected in the laying period, often at the peak of egg production or just befor production. A slight increase in mortality, decrease in feed intake and a drop in egg production can be observed. (Swayne 2020)

Figure 1 17-weeks old turkeys with an acute *O. rhinotracheale* infection, (M. Mägdefrau, Tierarztpraxis Mägdefrau)

Diseased turkeys show an edema of the lungs and uni- or bilateral pneumonia with fibrinous to purulent exudate. Often a fibrinous pericarditis and airsacculitis is present. Sometimes, swelling of the liver and the spleen can be noticed. Furthermore, a fibrinous to purulent arthritis is a frequent pathological finding in turkeys. The tarsal joint is often affected. (Swayne 2020; Siegmann 2011)

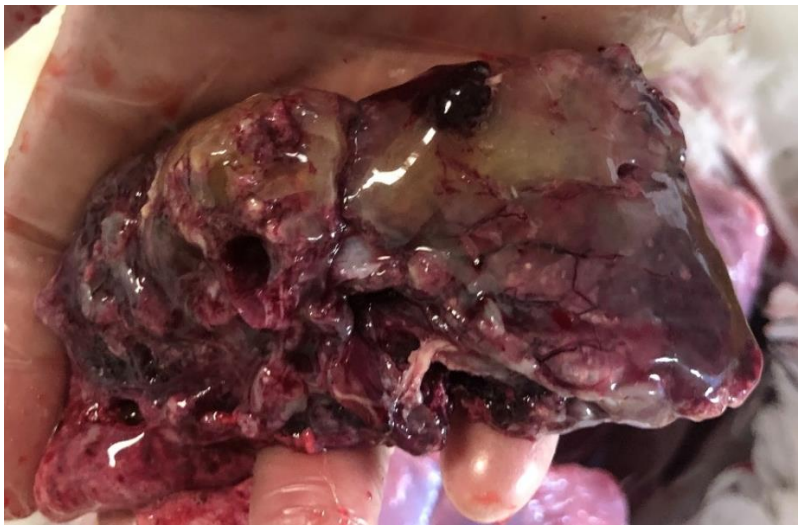


Figure 2 lung with fibrinous to purulent pneumonia of a 17-week-old turkey with *O. rhinotracheale* infection (M. Mägdefrau, Tierarztpraxis Mägdefrau)

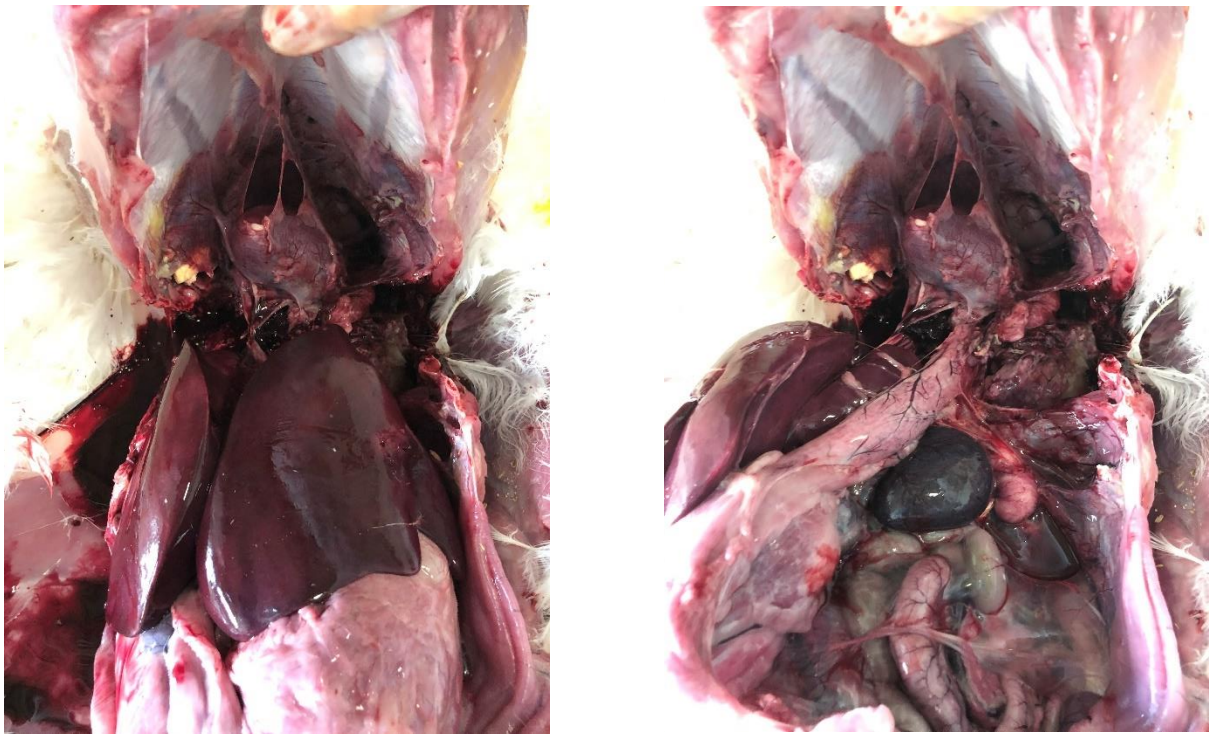


Figure 3 Carcass of a deceased 17-week-old turkey with *O. rhinotracheale* infection presenting pericarditis, fibrinous to purulent pleuritis, swelling of liver and spleen (left: organs *in situ*; right: liver and parts of gastrointestinal tract removed) (M. Mägdefrau, Tierarztpraxis Mägdefrau)

Histological lesions are noticed in the most affected organs, the lungs, air sacs and pleura. It is possible to see collections of fibrin mixed with macrophages and heterophils in air capillaries and parabronchi. Many necrotic areas throughout the lungs, pleura and air sacs can be seen. The pleura and air sacs can be thickened and edema, fibrin and heterophilic infiltrates can be found. (Swayne 2020)

2.3 Diagnosis, treatment and prophylactic measures

A presumptive diagnosis is often possible based on the clinical symptoms in combination with typical pathological lesions. For a final diagnosis however, the isolation and identification of *O. rhinotracheale* is required. Tracheal swabs from live animals or samples of lung tissue and air sacs taken during necropsy are best suited to isolate the pathogen. (Siegmann 2011)

O. rhinotracheale can be cultivated as described in 2.1. Because of the slow growth there is the possibility of overgrowth by other bacteria such as *E. coli*, *Proteus* sp. or *Pseudomonas* sp. (Swayne 2020). To prevent overgrowth blood agar including gentamycin can be used due to the frequent gentamycin resistance of *O. rhinotracheale* (Swayne 2020). Further diagnostic techniques include PCR (Swayne 2020) and MALDI-TOF (Alispahic et al. 2021).

Serology can be a useful tool for monitoring the whole flock. Different ELISA systems have been developed to detect antibodies against all known serotypes of *O. rhinotracheale*. (Swayne 2020)

In general, *O. rhinotracheale* infections can be treated with antibiotics, such as amoxicillin or tetracyclines (Swayne 2020). However, many *in vitro* antibiotic susceptibility tests from around the globe have shown limited susceptibility to many antibiotic substances (Swayne 2020; Siegmann 2011; Malik et al. 2003; Churria et al. 2016; Peña-Vargas et al. 2016; Szabó et al. 2015). The possible resistance include resistance against aminopenicillins, tetracyclines, fluoroquinolones, aminoglycosides, macrolides and diaminopyrimidines (Swayne 2020; Barbosa et al. 2019).

To prevent the disease strict biosecurity measures and thorough cleaning and disinfection are recommended (Siegmann 2011). In a farm which has already been infected it is likely that *O. rhinotracheale* becomes a frequent problem (Swayne 2020). This is especially true for multiple-age farms and areas with high poultry density (Swayne 2020).

Vaccines can be used to prevent *O. rhinotracheale* infections. Inactivated vaccines are available for broiler breeders, which induce an immune protection through maternal antibodies. Furthermore, the use of autogenous vaccines is possible. (Siegmann 2011)

3 Material and methods

3.1 Bacterial isolates

From 2002 to 2021 a total of 66 field strains of *Ornithobacterium rhinotracheale* were isolated from outbreaks in Austria and stored at -80°C (Table 1). These outbreaks occurred in 42 farms in different areas of Austria. All farms were marked to enable identification of strain origin. In farms 4, 8, 9, 11, 19, 24, 32, 34 and 35 isolates were obtained from multiple outbreaks within one year. Strains from farms 1, 2, 11, 18, 21, 24, 26, 27, 31 and 33 were isolated from several outbreaks over the years.

All strains were stored at -80 °C in 2 ml of 40 % glycerol/10 ml Brain Heart Infusion Broth (Oxoid©, ThermoFisher Scientific, Austria). In order to study the collected isolates, they were thawed and cultivated on blood agar (Columbia agar containing 5 % sheep blood, BioMeriéux, Austria) at 37 °C for 48 h under microaerophilic conditions (Genbox microaer©, BioMèrieux, Vienna, Austria).

3.2 Phenotypic characterization

After cultivation all strains were examined by various phenotypic aspects. The examined properties were size of colony, homogeneity of colony size, colour and nature of surface, hemolysis and possible abnormalities. To enable a comparison between strains and determine possible correlations between phenotypic properties and resistance against antibiotics, all investigated characteristics were categorized.

After 48 hours of incubation under microaerophilic conditions on blood agar the strains were examined and classified for different phenotypic properties. The examined properties were size, colour of appearance, hemolysis and heterogeneity of colonies. Regarding the size of colony, the strains were divided into five groups (table 1). Due to the high heterogeneity of O. *rhinotracheale* colonies, in doubt the smallest colonies of the strain determined the classification.

Table 1 *Categories (group) for O. rhinotracheale colonies regarding their size*

group:	size:	description:
group 1	< 0.3 mm	all strains, which colonies are mostly under 0.3 mm (including mostly pin sized colonies)
group 2	0.3 mm – 0.7 mm	all strains, which colonies are bigger than 0.3 mm and mainly smaller than 0.7 mm
group 3	0.7 mm – 1.2 mm	all strains with colonies mainly ranging from 0.7 mm to 1.2 mm; some colonies may be slightly bigger/smaller
group 4	1.2 mm – 2.0 mm	all strains, which colonies are all bigger than 1.2 mm but mostly smaller than 2.0 mm
group 5	> 2.0 mm	all strains with most colonies over 2.0 mm

In terms of their colour and appearance the strains were divided into three categories (table 2).

Table 2 *Categories (colour of colonies) for O. rhinotracheale colonies regarding their colour*

colour of colonies:	description:
grey	all strains, which are of grey colour without much deviation; includes dark grey, light grey, shiny grey, mat grey
transparent	all strains, that have a significant transparent component; often combined with a greyish tone
light red	all strains, which feature a significant reddish or pink colour

Regarding the hemolysis the strains were divided into three groups (table 3).

Table 3 *Categories (group) for O. rhinotracheale colonies regarding their hemolysis*

group:	description:
no hemolysis	No signs of hemolysis are visible
α-hemolysis	no continuous β-hemolysis, in some places incomplete “green” hemolysis
β-hemolysis	mixture of α- and β-hemolysis with emphasis on β-hemolysis or full β-hemolysis

Regarding the heterogeneity of the colonies the strains were divided into three groups (table 4).

Table 4 *Categories (group) for O. rhinotracheale colonies regarding their heterogeneity*

group:	size variation:	Description:
No heterogeneity	< 0.5 mm	all colonies are very similar and do not differ significantly in size
Little to medium heterogeneity	0.5 mm – 1.0 mm	colonies differ between 0.5 mm and 1 mm in size, but most colonies lie closely around an average
High heterogeneity	> 1.0 mm	colonies differ more than 1.0 mm in size and distribution is spread over the whole size range without a noticeable accumulation around an average

Table 5 *Strains investigated sorted by date of isolation with farm and region of origin (LA: Lower Austria, UA: Upper Austria, B: Burgenland, Sa: Salzburg)*

Strain name	Farm	Region
02/103	1	LA
02/301	2	LA
02/658	3	LA
03/525	4	LA
03/526-3	4	LA
03/530	5	UA
03/638-3	2	LA
03/2652	6	LA
03/3567	7	UA
04/0372-1	8	B
04/0372-3	8	B
04/0471-1	9	LA
04/4559-1	9	LA
04/4559-2	9	LA
04/1260	10	UA
04/1972-2	11	LA
04/3032	12	UA
04/3086	13	LA
04/4018	14	UA
04/4426-1	15	LA
04/4519-2	17	LA
05/0091	1	LA
06/5549	18	UA
06/8251-2	19	B
06/8251-3	19	B
06/8251-5	19	B
09/8720	31	LA
09/10711	38	LA
09/16066	22	LA
10/0047	18	UA
10/3279	28	LA
10/11996	24	LA

10/11997	24	LA
10/15448	39	LA
11/35	40	LA
11/3656	24	LA
12/0577	11	LA
12/18482	27	UA
12/19777	33	B
13/0573	18	UA
13/00771	25	B
13/1156	26	LA
14/0574	42	LA
14/2621	27	UA
14/20273	11	LA
15/14086	18	UA
15/25053	21	LA
16/0975	31	LA
16/1621	1	LA
16/2802	11	LA
16/19386	11	LA
16/22481	27	UA
16/22784	24	LA
17/10229	21	LA
17/15431	31	LA
17/16762	44	UA
18/3857	37	UA
19/8256	35	UA
19/8886	35	UA
19/24041	34	LA
19/27200	34	LA
20/21866	32	Sa
20/21867	32	Sa
21/5879	33	B
21/6763	26	LA
21/15252	37	UA

3.3 Matrix Assisted Laser Desorption - Time of Flight Mass Spectrometry

A total of 37 of the collected strains were previously analysed and characterized by MALDI-TOF (Alispahic et al. 2021). The remaining 29 isolates were investigated within the present study by MALDI-TOF MS as well.

The direct transfer method of the isolates was performed by applying one to two single colonies on the steel target plate by using disposable 1 µl loops (loops 1 µl hard, Copan Italia spa, Italy). Afterwards, 1 µl matrix solution (alpha-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid) was spotted on top of each dried sample and left to dry again. The parameter settings for the Microflex LT instrument were as follows: IS1, 20.08 kV; IS2, 16.60 kV; lens, 7.00 kV; detector gain, 2974 V. Two hundred and forty laser shots in 40 shot steps (in the linear, positive ion mode with a 60 Hz nitrogen laser from different positions of the target spot) were summarized automatically with the AutoXecute (MBT AutoX method) acquisition control software (Flex control 4©, Bruker Daltonics GmbH, Germany). For automated data analysis, the raw spectra for unknown bacteria were processed using MALDI Biotyper software (Bruker Daltonics GmbH, Germany) with the default settings. The software performs smoothing, normalization, baseline subtraction, and peak picking, thereby creating a list of the most significant peaks (m/z values) of the spectrum. For species identification, the MALDI Biotyper output is a log (score) in the range of 0 to 3.0, computed by comparison of the peak list for an unknown isolate with the reference Main Spectra (MSP) in the reference database as well as the in-house reference database according Alispahic et al. (2021). A MALDI score between 1.7 and 2.0 represents genus identification, while a MALDI score above 2.0 represents identification at species level. Anything less than 1.7 was rated as non-identifiable by the software.

3.4 Antibiotic susceptibility testing

The antibiotic susceptibility testing was done by microdilution applying the MICRONAUT-S system (MICRONAUT-S©, MERLIN Gesellschaft für mikrobiologische Diagnostika mbH, Germany). This is a susceptibility testing system, which is based on the rehydration of antibiotics with a standardized bacteria suspension, resulting in different concentrations of the antimicrobial substances (table 6). Through the ascending concentrations of each antibiotic typical to microdilution procedures, it is possible to determine the minimal inhibitory

concentration (MIC) according to CLSI standards (CLSI 2008). The growth results are read photometrically and evaluated by the MICRONAUT Software. The MICRONAUT system shows high concordance with reference gradient diffusion methods (MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH; Cordovana and Ambretti 2020; Baquer et al. 2021; Pfennigwerth et al. 2019)

Table 6 *Antibiotic substances and their concentrations in the MICRONAUT-S system*

class	Antibiotic substance	Concentration (µg/ml)									
penicillin	amoxicillin	4	8	16	32						
	amox./clavulanic acid	4/2	8/4	16/8	32/16						
	ampicillin	0.25	0.5	1	2	4	8	16			
	oxacillin	0.25									
cephalosporin	cefazolin (1st gen.)	2	4								
	cefoxitin (2nd gen.)	4									
	cefotaxim (3rd gen.)	0.25	0.5	1	2	4	8	16	32		
	ceftazidime (3rd gen.)	0.25	0.5	1	2	4	8	16	32		
chloramphenicol	chloramphenicol	4	8	16	32						
polypeptide	colistin	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16
quinolone	enrofloxacin	0.125	0.25	0.5	1	2					
	nalidixic acid	4	8	16	32	64					
aminoglycoside	gentamycin	1	2	4	8	16					
	neomycin	4	8	16	32						
	streptomycin	8	16	32	64						
carbapenem	imipenem	1	2	4							
tetracycline	tetracycline	0.25	0.5	1	2	4	8	16			
diaminopyrimidine/ sulfamethoxazole	trimethoprim	8	16								
	sulfamethoxazole	256	512								
	trimeth./sulfameth.	0.5/9.5	1/19	2/38	4/76						
macrolide	tyosin	1	2	4	8	16					

The company, which developed the MICRONAUT System provides detailed recommendations on the preparation of the inoculum for a number of bacteria (MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH). For *O. rhinotracheale* no such guidelines exist. Since

there are no official recommendations and no widely accepted publications on the testing of antibiotic susceptibility of this bacteria with MICRONAUT-S, a variety of parameters was tested to determine the most suitable one.

The tested parameters were culture medium, concentration of bacteria suspension, reading software and time of measurement. The strains were always cultivated under microaerophilic conditions (Genbox microaer©, BioMèrieux, Vienna, Austria) at 37 °C. All tests were conducted on three different strains of *O. rhinotracheale*, which differed in their phenotypic appearance. The strains used for these tests were 02/103, 02/301 and 02/658.

In the first cycle of testing the existing recommendations for the Micronaut system for gram negative, gram positive and fastidious bacteria, were used. A McFarland 0.5 bacteria suspension was made for each strain of. *O. rhinotracheale*. The used culture media were Mueller-Hinton broth (Oxoid™ Mueller-Hinton Broth CM0405, ThermoFisher Scientific, Austria) with 50µl of bacteria suspension, Mueller-Hinton broth with 2.5 % lysed horse blood (Oxoid© Laked Horse Blood, Thermo Scientific, Germany) with 200 µl of bacteria suspension and H-broth (MICRONAUT H-Medium, Merlin Gesellschaft für mikrobiologische Diagnostika mbH, Deutschland) with 50 µl and 200 µl of bacteria suspension respectively. The suspensions were transferred into the wells of the test plates. After 24 h and 48 h of cultivation the growth was measured and MIC values were interpreted when available. The analysis was done with the recommended software settings (R60 for MH-broth; H60 for MH-broth + 2.5 % blood; H60 for H broth).

In the second cycle of testing McFarland 1, 2 and 3 bacteria suspensions were made for each of the three strains. 200 µl respectively were transferred in Mueller-Hinton broth and Mueller-Hinton broth with 2.5 % lysed horse blood. After 24 h, 48 h, 72 h and 96 h growth was measured and MIC values were interpreted when available. The analysis was done with different software settings (Rx, Hx, Wx) for both broths.

In the third cycle of testing McFarland 0.5 and 2 bacteria suspension were made for each of the three strains. 200 µl of every suspension was transferred in Lysogeny broth (Invitrogen© LB Broth Base, ThermoFisher Scientific, Austria) and Lysogeny broth with 2.5 % lysed horse blood. After 24 h and 48 h growth was measured and MIC values were interpreted when available. Analysis was done with different software settings (Rx, Hx, Wx) for both broths.

Furthermore, the antibiotic susceptibility of *O. rhinotracheale* strains was analysed and a variety of factors and developments investigated. In addition to the analysis of all tested

antibiotic substances, for some investigations only the data of antibiotics relevant for the use in Austrian poultry and farm animals in general was used. Frequently used antibiotics for poultry in Austria were amoxicillin, neomycin, enrofloxacin, tetracycline, tylosin, colistin, trimethoprim, sulfamethoxazole and the combination of trimethoprim and sulfamethoxazole. Because of their use in other farm animals in Austria, the cephalosporins cefazolin, cefoxitin, cefotaxime and ceftazidime were included as well (Bundesamt für Sicherheit im Gesundheitswesen 2021).

3.5 Statistics

All calculations were done in Excel (Excel©, Microsoft Corporation, United States of America). The raw data was delivered by the MICRONAUT Software and manually transferred to Excel. All graphs were plotted with Excel, using bar charts, grouped bar charts and XY-Scatter plots.

4 Results and discussion

4.1 Phenotypic characterization

4.1.1 Examination of phenotypes

4.1.1.1 Size and heterogeneity of colonies

O. rhinotracheale colonies are generally described in literature as small and heterogeneous in terms of size (van Empel et al. 1997; Zahra et al. 2013; Swayne 2020). They usually range from 1 mm to 3 mm with large deviation within one strain(Zahra et al. 2013) (van Empel et al. 1997), but also so-called “pinpoint” colonies with diameters noticeably below one millimetre have been described (Zahra et al. 2013). These pinpoint colonies may also be referred to as small colony variants or SCVs (Proctor et al. 2006; Zahra et al. 2013).

To gain an overview of the occurrence of different *O. rhinotracheale* phenotypes in Austria, all 66 strains were examined and divided into different size-categories. As already described in chapter 3.1 five different categories were used. SCVs are usually defined through their colonies having about one-tenth the size of the normal sized colonies (Proctor et al. 2006). For *O. rhinotracheale* with its already small colonies this would imply SCVs to be smaller than 0.3 mm. However, there is no such strict classification for *O. rhinotracheale* yet, with some literature dividing colonies only in normal (1 – 3 mm) and pinpoint without exact size definition (Zahra et al. 2013). To be able to distinguish better between the different sizes of colonies, which are below 1 mm, three of the five categories were established to cluster the strains with colonies under 1 mm. Due to the high heterogeneity of size even within one strain, the smallest colonies of a strain got most of the attention for classifying. Only if the vast majority of colonies was in a bigger category than the smallest ones, the whole strain was classified in this bigger size group.

The first category includes all strains with colonies with a diameter mainly ≤ 0.3 mm. These colonies were mostly so small that an exact measurement of size was not possible. These colonies are truly pinpoint-sized (Proctor et al. 2006) and differ optically very clearly from all other categories. The second category included strains with colonies ranging from 0.3 mm to 0.7 mm. These colonies are also clearly below one millimetre in size, which is usually the lower boundary in size descriptions (Swayne 2020; Siegmann 2011) and thus might sometimes be referred to as pinpoint colonies (Zahra et al. 2013). They are significantly bigger than the

previously described colonies. The third category includes all strains with colonies ranging around 1 mm. In this category there are many typical *O. rhinotracheale* phenotypes with the smallest colonies around 1 mm mixed with some bigger colonies. The fourth category includes also quite typical *O. rhinotracheale* colonies with sizes between 1.2 mm and 2 mm. The fifth category is reserved for all strains, which have mainly colonies > 2 mm and only some individual colonies are slightly smaller.

With this classification it is possible to distinguish in more detail between the usual colonies ranging from 1 mm to 3 mm in diameter and the smaller pinpoint like colonies.

From the 66 strains isolated between 2002 and 2021 10.61 % were classified in category 1, hence having colonies smaller than 0.3 mm (figure 4). 22.73 % of the strains had colonies between 0.3 mm and 0.7 mm and were classified as category 2. Most strains were classified as category 3, to which 31.82 % of all strains belonged. With 27.27 % the second most common category was category 4. The smallest category was number 5 with only 7.58 % of all strains having no or nearly no colonies smaller than 2 mm.

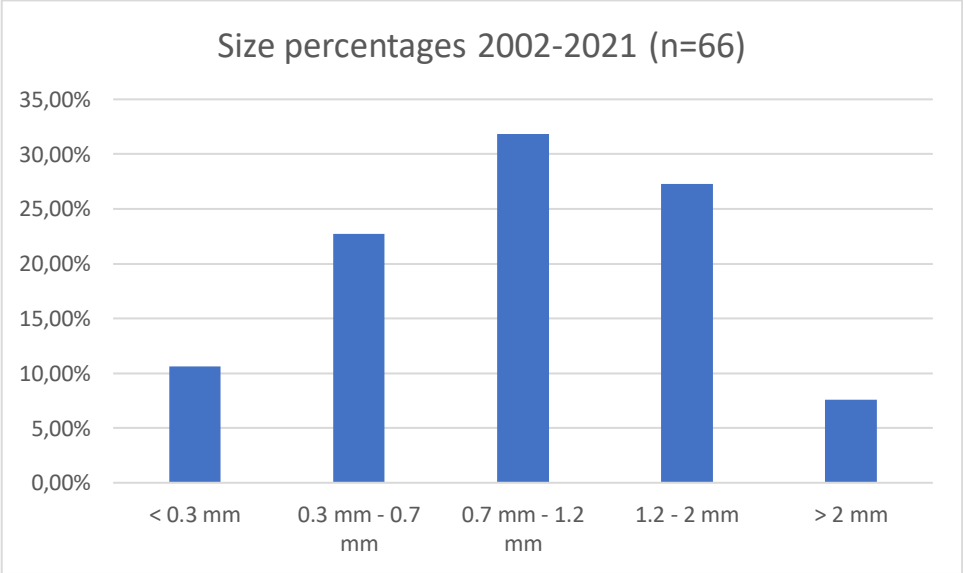


Figure 4 *Colony size of O. rhinotracheale strains*

In a study from China 18.5 % of the investigated strains were classified as SCVs and referred to as pinpoint colonies (Zahra et al. 2013). However, the comparability of their data may be limited because of the relatively small number of strains, with only 27 *O. rhinotracheale* isolates in total (Zahra et al. 2013).

When looking at the prevalence of colony sizes in different years, there are certain changes noticeable (figure 5). The strains were grouped into three time periods with approximately the same number of years and strains. Period 1 dates from 2002 to 2006 and includes 26 strains. The second period ranges from 2009 to 2015 and includes 21 strains. The third period spans from 2016 to 2021 and includes 19 strains.

From the first period to the last one the proportion of strains with small, “pinpoint” colonies (< 1.2 mm) rises considerably, while the number of strains with bigger colonies drops (figure 5). From 2002 to 2006 53.84 % of all strains had colonies smaller than 1.2 mm. From 2009 to 2015 66.67 % of the strains had colonies smaller than 1.2 mm. In the period from 2016 to 2021 with 78.95 % the majority of strains had colonies smaller than 1.2 mm, thus classified as “pinpoint-colonies”. However, this does not mean that in recent years only about 20 % of strains had colonies bigger than 1.2 mm, since many strains show at least some heterogeneity of colony size. Therefore, many strains may have pinpoint colonies as well as bigger ones.

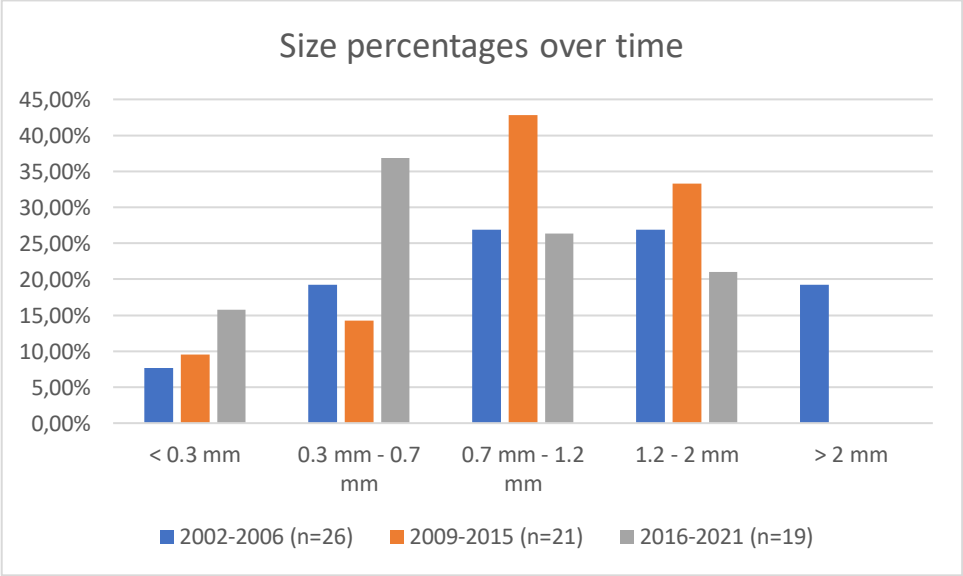


Figure 5 *Size distribution in percent of O. rhinotracheale colonies in different time periods*

It is often described that colonies of the same *O. rhinotracheale* strain vary significantly in size (van Empel et al. 1997) (Zahra et al. 2013). To confirm this for the 66 strains from Austria they were assessed regarding their heterogeneity. To analyse the results the strains were divided into three groups. The first group includes all strains with colonies identical in size. The second group includes strains with little to medium heterogeneity. This means that the colonies of one

strain differ in size up to around 1 mm, but most of the colonies are roughly about the same size. The third and last group includes strains, which colonies differ substantially in size. The colonies differ more than 1 mm and they are spread over the whole range of size without recognizable accumulation around a mean size.

From the 66 *O. rhinotracheale* strains collected between 2002 and 2021 48.48 % showed no heterogeneity, 37.88 % of the strains showed little to medium heterogeneity and 13.64 % showed a high heterogeneity (figure 6). For correct interpretation of this data, it is important to know, that these colonies originate from re-cultured pre-isolated and previously sub-cultured strains. Primary cultures often show more heterogeneity than subcultures (van Empel et al. 1997).

There were no big changes over the years. Only strains with high heterogeneity were more frequent when comparing the three time periods. Between 2002 and 2006 7.69 % of strains showed a high heterogeneity, while in the period of 2009 to 2015 14.29 % of all strains showed high variance in size and from 2016 to 2021 21.05 % of the strains were classified as being of high heterogeneity (data not shown).

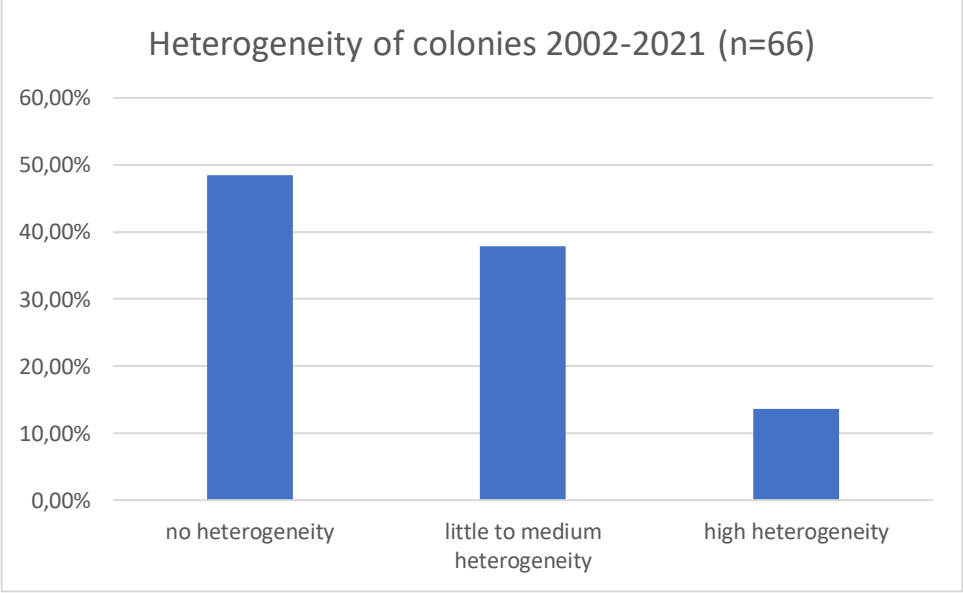


Figure 6 *Heterogeneity of colony sizes of O. rhinotracheale strains*

When studying the heterogeneity of the strains in more detail, different results depending on the main colony size of the strains can be noticed. The majority of strains with colonies smaller than 0.3 mm showed no heterogeneity (85.71 %). These strains grew very uniform, truly minuscule colonies (figure 7). Strains belonging to the category 0.3 – 0.7 mm show the most heterogeneity of all categories. These strains mostly form both very small and larger, classical sized colonies.

All larger categories show either no or little to medium heterogeneity. The proportions of all three categories above 0.7 mm are quite similar. Between 50 % and 60 % of the strains show no heterogeneity while 40 % to 50 % of the strains show little to medium heterogeneity.

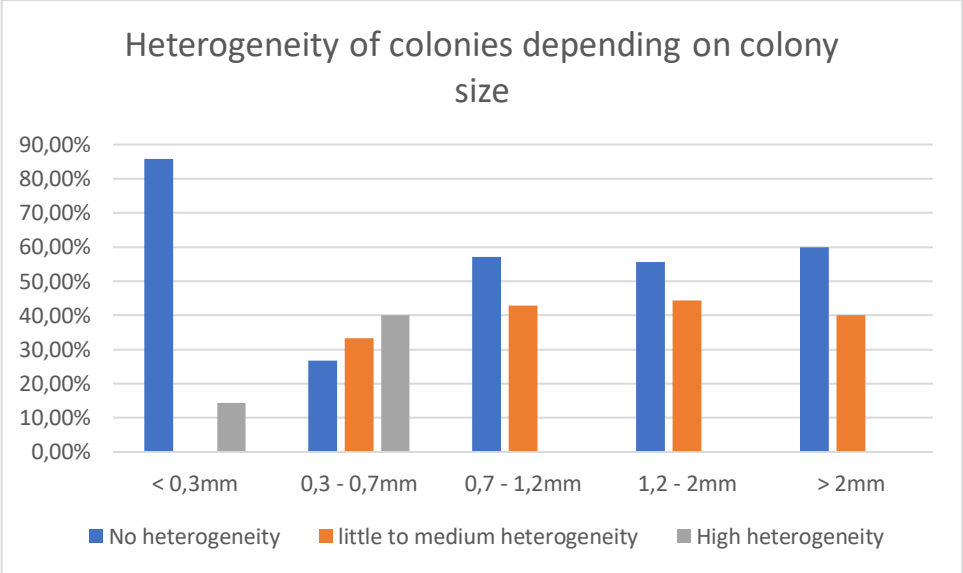


Figure 7 *Heterogeneity of colony size depending on the classification of the colony size*

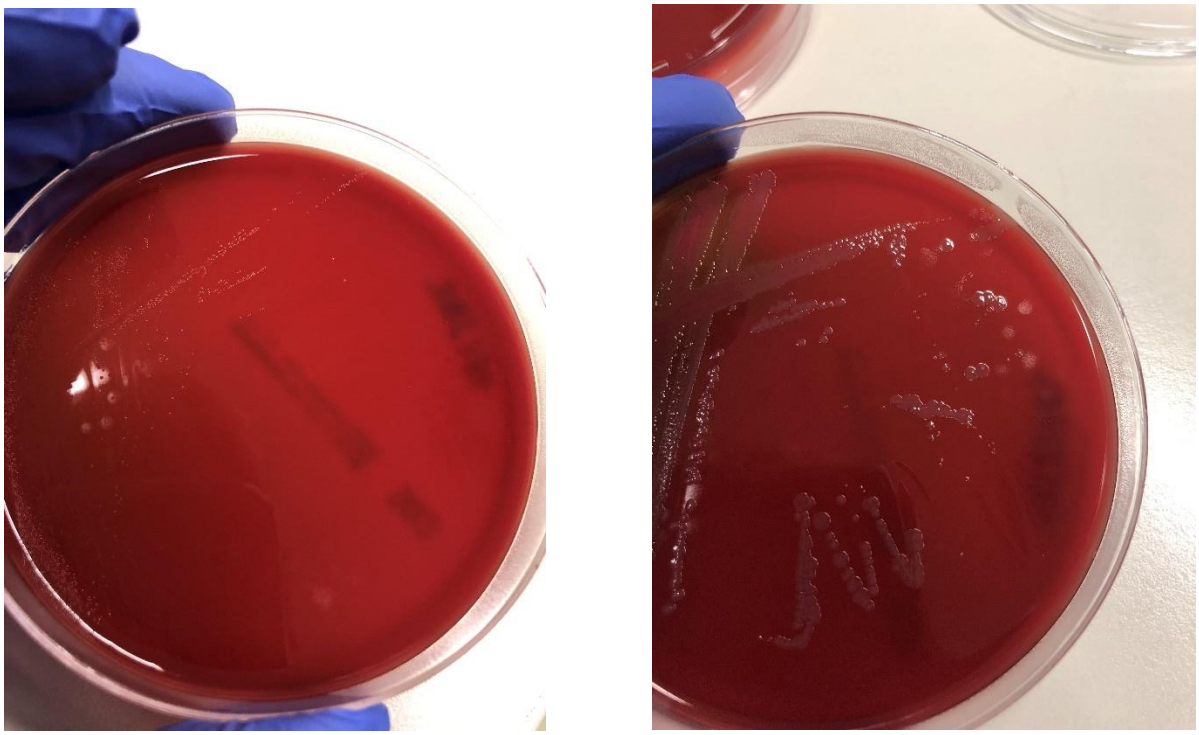


Figure 8 *O. rhinotracheale colonies of different strains (<0.3 mm colonies in left picture; >2 mm colonies in right picture) (M. Mägdefrau, Clinic for Poultry and Fish Medicine, Vetmeduni Vienna)*

4.1.1.2 Appearance and hemolysis of colonies

Regarding the colour of colonies and development of hemolysis *O. rhinotracheale* is quite variable, thus descriptions in literature are accordingly varying. Colonies are traditionally described as grey-white and opaque with no hemolysis (Hafez Mohamed Hafez 2002; Siegmann 2011; Swayne 2020). Sometimes more transparent colonies (Zahra et al. 2013) or colonies with a reddish colour (van Empel et al. 1997; Swayne 2020) are described. Especially more recently also *O. rhinotracheale* isolates capable of hemolysis are described (Zahra et al. 2013; Tabatabai et al. 2010; Walters et al. 2014).

In order to compare the 66 Austrian strains to the international literature, their colonies were examined and classified as follows. Regarding their colour, the colonies are divided into three groups. Group 1 includes all grey to grey-white and mostly opaque colonies, thus represents the traditional description. Group 2 is for all strains with colonies that are significantly less opaque or even nearly transparent. Group 3 includes all strains with reddish colonies.

Of the 66 strains from Austria 57.58 % were categorised as “grey”. They match the typical description of *O. rhinotracheale* colonies. 22.73 % of the strains were noticeably less opaque than other colonies and thus assigned to the group “transparent”. They vary from less opaque to nearly completely transparent. Of course, the smaller the colonies are, the more transparent they appear independent of their true colour, because the smaller bacterial mass filters less light. About a fifth of the strains (19.70 %) develops colonies with a reddish colour or a reddish glow. They differ visually significantly from the other *O. rhinotracheale* colonies.

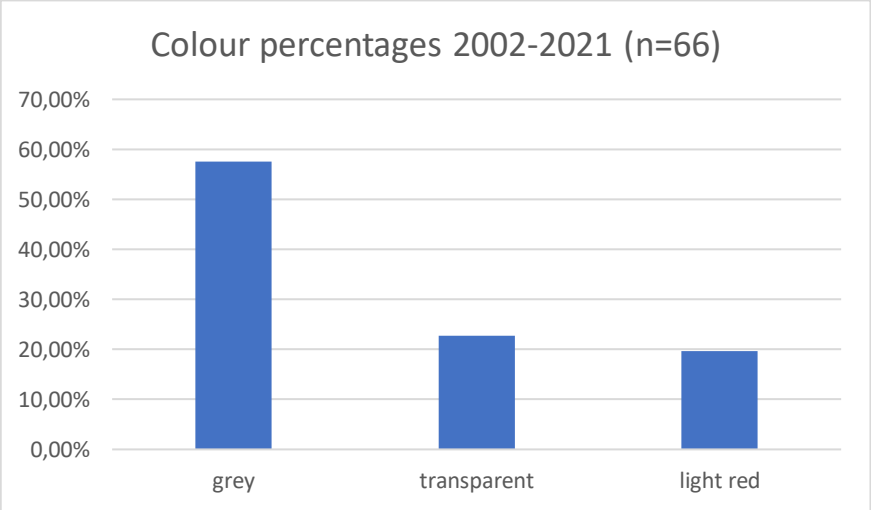


Figure 9 *Categories of colour (%) applied to differentiate O. rhinotracheale strains*

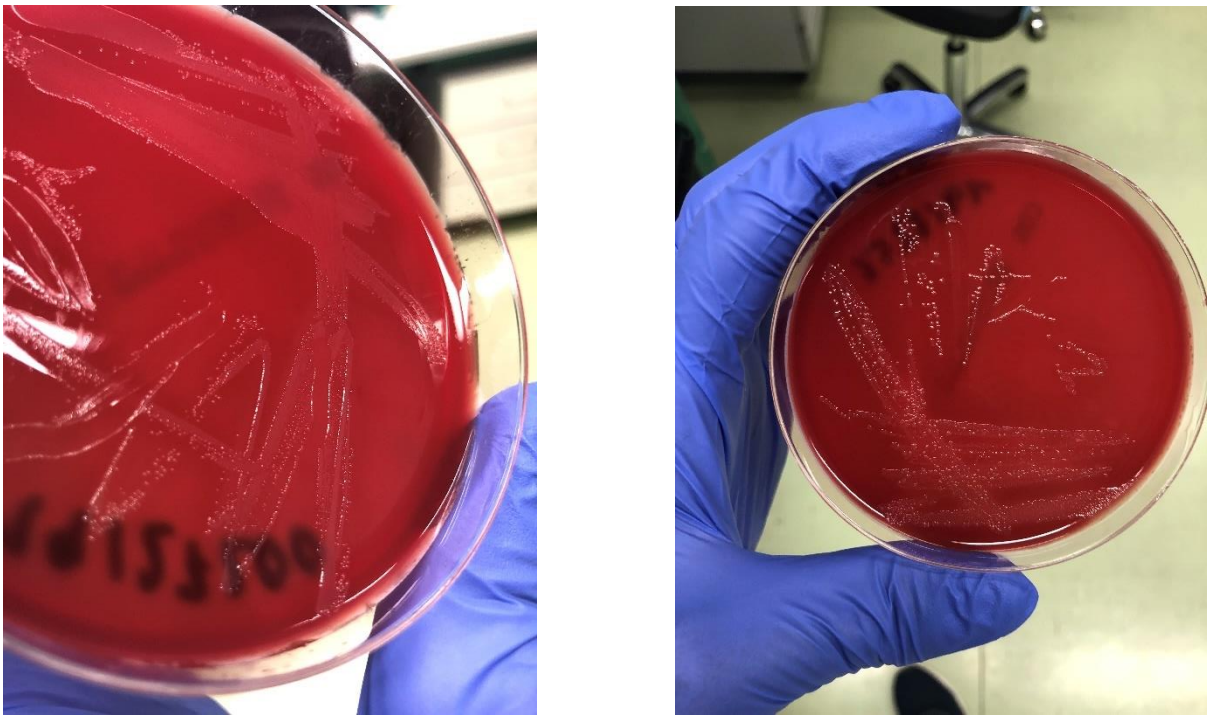


Figure 10 *O. rhinotracheale colonies of different strains (colonies with reddish colour in left picture; transparent colonies in right picture) (M. Mägdefrau, Clinic for Poultry and Fish Medicine, Vetmeduni Vienna)*

To distinguish between the different abilities to cause hemolysis of *O. rhinotracheale* strains, they got divided into three different groups (figure 11). The first group shows no hemolysis. The second group includes all strains with some form of hemolysis, but no continuous β-hemolysis. In this group many strains with incomplete, green hemolysis are found. Therefore, the group is called α-hemolysis. The last group includes all strains with predominant β-hemolysis.

Of all 66 collected strains 39.39 % showed no hemolysis. 43.94 % could be attributed to the α-hemolysis. 16.67 % of the strains developed a β-hemolysis. These findings are in agreement with results from a study in the United States of America, where most isolates showed some form of hemolysis on sheep blood agar and a smaller portion of the strains was able to develop β-hemolysis (Tabatabai et al. 2010). In the same study they found 6 peptides in *O. rhinotracheale* isolates with high similarities to known hemolytic leukotoxins from other bacteria (Tabatabai et al. 2010).

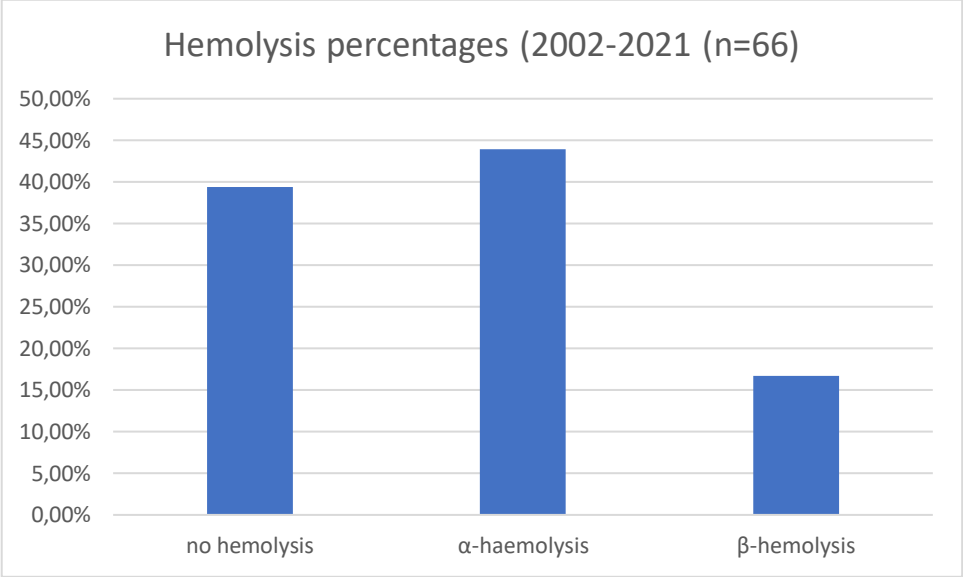


Figure 11 *Hemolysis (%) of all strains*

Since a frequent appearance of β-hemolysis is discussed in recent literature, the development of β-hemolysis in Austrian strains over time was examined. Again, the strains were divided into the three groups (2002-2006, 2009-2015 and 2016-2021). Between 2002 and 2006 only 7.69 % of the strains were able to develop a β-hemolysis. In the years 2009 to 2015 19.05 % of the strains showed β-hemolysis. From 2016 to 2021 the share of strains causing β-hemolysis rose to 26.32 %.

Linking β-hemolysis with colony size of the strain, differences are recognisable. From the strains with colonies smaller than 0.3 mm 28.57 % showed β-hemolysis. The strains with colonies sized between 0.3 mm and 0.7 mm showed no ability to cause β-hemolysis at all. From the strains with colonies ranging in size from 0.7 mm to 1.2 mm 28.57 % developed β-hemolysis. No β-hemolysis was found in the group of strains with the biggest colonies. From these findings no clear correlation is visible. (Zahra et al. 2013) reported, that SCVs of *O. rhinotracheale* are not able to develop β-hemolysis.

While strains with hemolytic abilities are often said to be more pathogenic, this may not be true for *O. rhinotracheale*. An experimental comparison of infections with hemolytic and nonhemolytic *O. rhinotracheale* strains showed, that non-hemolytic strains are more virulent and cause more severe lesions. (Walters et al. 2014)

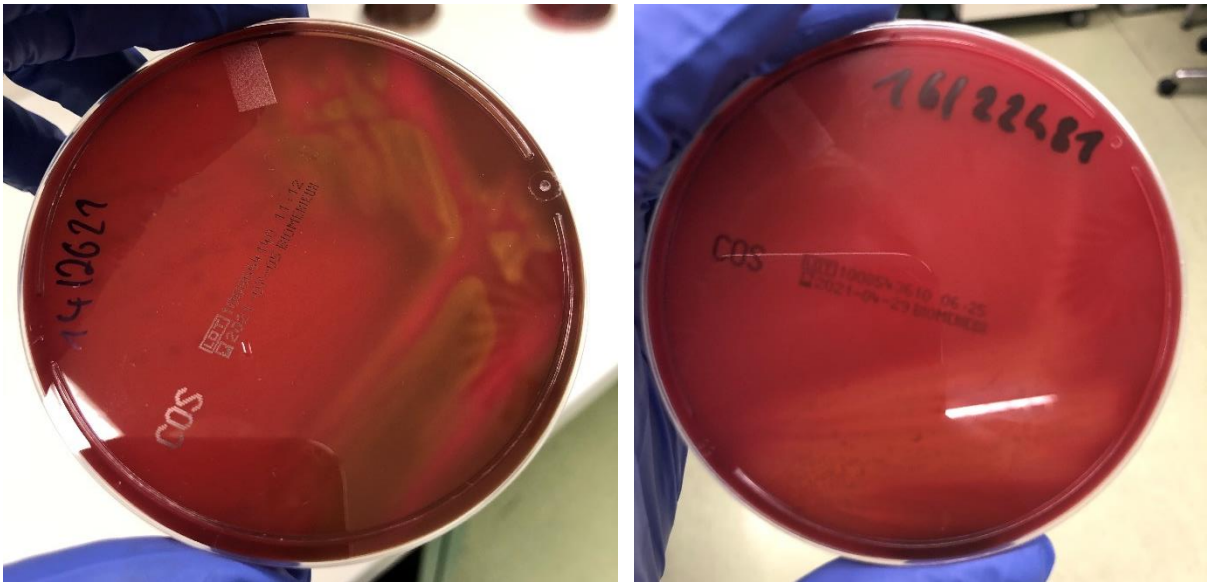


Figure 12 *O. rhinotracheale colonies of different strains (incomplete hemolysis in left picture; complete hemolysis in right picture)-(M. Mägdefrau, Clinic for Poultry and Fish Medicine, Vetmeduni Vienna)*

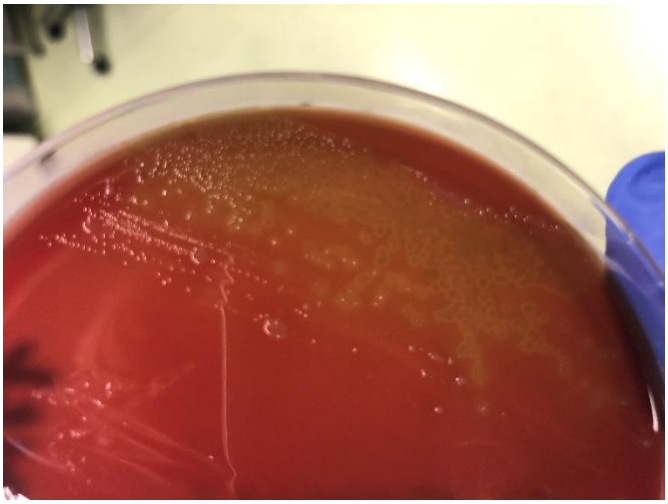


Figure 13 *O. rhinotracheale colonies with visible hemolysis around each colony (M. Mägdefrau, Clinic for Poultry and Fish Medicine, Vetmeduni Vienna)*

4.2 MALDI TOF MS

It was shown in previous studies, that MALDI-TOF MS is a suitable technology to distinguish between pathogens with relevance for poultry on a species level, including *O. rhinotracheale* (Alispahic et al. 2011; Alispahic et al. 2014; Alispahic et al. 2021). Therefore, 29 strains, that have not been examined with MALDI-TOF MS previously, were investigated by MALDI-TOF MS (table 7). All strains were identified on a species level. 26 of the 29 strains had a MALDI-TOF MS score value of 2.30 or higher, hence being classified as “highly probable species identification”. The three remaining strains had a score between 2.00 and 2.29 and have therefore been classified as “secure genus identification, probable species identification”. There were no results with a score <2.00, what would have been identification on genus level.

Since it was previously shown, that serotypes F, K and M get MALDI-TOF MS score values of below 2.00 or below 1.70 (Alispahic et al. 2021), it is probable that none of the strains in the present study belong to the serotypes F, K and M.

Table 7 *MALDI Score values of O. rhinotracheale strains isolated from Austrian turkey flocks*

Strain Name	MALDI Score Value	Description
09/08720	2.40	highly probable species identification
09/10711	2.49	highly probable species identification
09/16066	2.36	highly probable species identification
10/00047	2.42	highly probable species identification
10/03279	2.51	highly probable species identification
10/15448	2.32	highly probable species identification
11/00035	2.37	highly probable species identification
11/03656	2.44	highly probable species identification
12/00577	2.47	highly probable species identification
12/18482	2.43	highly probable species identification
12/19777	2.48	highly probable species identification
14/00574	2.29	secure genus identification, probable species identification
14/20273	2.39	highly probable species identification
15/14086	2.30	highly probable species identification
16/02802	2.18	secure genus identification, probable species identification
16/19386	2.37	highly probable species identification
17/10229	2.52	highly probable species identification
17/15431	2.47	highly probable species identification
17/16762	2.28	secure genus identification, probable species identification
18/03857	2.30	highly probable species identification
19/08256	2.33	highly probable species identification
19/08886	2.43	highly probable species identification
19/24041	2.45	highly probable species identification
19/27200	2.48	highly probable species identification
20/21866	2.38	highly probable species identification
20/21867	2.30	highly probable species identification
21/05879	2.40	highly probable species identification
21/06763	2.38	highly probable species identification
21/15252	2.38	highly probable species identification

4.3 Evaluation of antibiotic susceptibility testing with Micronaut-S

4.3.1 Evaluation of antibiotic susceptibility test protocols

The Micronaut-S software differentiates three different graduations of too little growth (in ascending order: “no growth”, “too little growth, check control visually” and “too little growth”). As soon as the photometer detects enough growth in the positive control, the software analyses the measurements and delivers MIC values. To allow comparison of bacterial growth, these four possible results will be aligned with a specific score from “0” (“no growth”), “1” (“too little growth, check control visually”), “2” (“too little growth”) to “3” (MIC values delivered). The required score is the number of strains (n) x 3, since a valid method should deliver constant growth. In general, the higher the score, the better the method. Therefore, an applicable method must always achieve the highest possible score of 3, because only then MIC testing is valid. Therefore, a higher score might indicate a better growth, but if it is not the maximum score it is not sufficient.

None of the recommended methods proved suitable to support sufficient growth of *O. rhinotracheale*, when starting with a maximum of 200 µl McFarland 0.5 bacteria suspension (table 8). With Methods 2, 3 and 4 some growth was noticed and after 48 h the results were slightly better compared to 24 h. In consequence a higher number of colony forming units (CFU) in the suspension at the beginning or a longer time of incubation might lead to valid results. Therefore, in test cycle 2 the influence of a higher number of CFU and longer incubation time were evaluated (table 9).

Table 8 *Results test cycle 1*

method	highest possible score	score after 24h	score after 48h
50 µl in MH broth	9	0	0
200 µl in MH broth + 2.5 % lysed horse blood	9	5	6
200 µl in H-broth	9	4	4
50 µl in H-broth	9	3	4

Table 9 *Results test cycle 2*

method	highest possible score	24h	48h	72h	96h
200 µl in MH broth + 2.5 % lysed horse blood Mc Farland 1	9	6	6	6	6
200 µl in MH broth + 2.5 % lysed horse blood Mc Farland 2	9	5	6	6	6
200 µl in MH broth + 2.5 % lysed horse blood Mc Farland 3	9	6	6	6	6
200 µl in H-broth Mc Farland 1	9	5	4	4	4
200 µl in H-broth Mc Farland 2	9	5	4	4	4
200 µl in H-broth Mc Farland 3	9	6	4	5	4

None of the tested methods was able to support sufficient growth of *O. rhinotracheale*. In addition, there seems to be no positive correlation between growth and incubation time nor with the number of colony forming units at the beginning. Hence, the results from cycle two show that neither the number of CFU at the starting point nor the incubation time were the critical negative variables.

Therefore, another broth, namely Lysogeny broth, which is a nutritionally rich media for growth of fastidious bacteria, was tested in cycle three. In the test lysogeny broth and lysogeny broth with 2.5 % lysed horse blood were used. In addition, bacteria suspensions with McFarland 0.5 and McFarland 2 were used for both broths, to determine any difference because of the amount of colony forming units at the beginning with Lysogeny broth.

Table 10 *Results test cycle 3*

method	Highest possible score	24h	48h
Lysogeny broth McFarland 0.5	9	4	5
Lysogeny broth McFarland 2	9	5	5
Lysogeny broth + 2.5 % lysed horse blood, McFarland 0.5	9	9	9
Lysogeny broth + 2.5 % lysed horse blood, McFarland 2	9	9	9

The results from cycle 3 (table 10) show a significant difference to the previously used broths and highlights the need to use a combination of a nutritious media and lysed horse blood to enable growth of *O. rhinotracheale*. Lysogeny broth without lysed horse blood was not able to support enough growth to enable measurable MIC results. The number of colony forming units at the beginning, hence a McFarland value of 0.5 or 2, seems to be not relevant in this setting. In Lysogeny broth with 2.5 % lysed horse blood *O. rhinotracheale* showed strong and consistent growth. For all three strains it was possible to measure valid MIC results with Micronaut-S test panels after 24 h and 48 h. Therefore, Lysogeny broth with 2.5 % lysed horse blood was the first method used in this study that fulfilled the necessary requirements.

According to the manufacturer of Micronaut-S the generally recommended McFarland concentration would be 0.5 (MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH). For fastidious bacteria the incubation time is recommended to be 48 h (MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH). To decide, whether there are any reasons not to follow this protocol, the different MIC results were compared and differences were evaluated.

A total of 23 antimicrobial substances were tested on each of the three strains and their different suspensions after 24 h and 48 h.

In table 11 the accumulated number of “sensitive”, “intermediate” and “resistant” results of all three strains for each protocol is shown. Each number is calculated by addition of e.g. “sensitive” results of strain 1, strain 2 and strain 3. In the last line the amount of deviations in the 23 tested antimicrobial substances when compared to the recommended setting is shown.

Table 11 *Test cycle 4 McFarland and incubation time*

	McFarland 0.5; 48 h (recommended)	McFarland 0.5; 24 h	McFarland 2; 24 h	McFarland 2.0; 48 h
Total number of sensitive results	**39**	41	40	38
Total number of intermediate results	**4**	7	5	4
Total number of resistant results	**26**	21	24	27
Number of different resistant results compared to McFarland 0.5; 48 h	*I*	5	2	1

With the recommended protocol of McFarland 0.5 and 48 h of incubation time the results for the first strain were 10 x sensitive, 2 x intermediate and 11 x resistant. The protocol with the same McFarland concentration of 0.5 but only 24 h of incubation time led to one more sensitive result (trimethoprim) and two more intermediate results (cefotaxim and nalidixic acid). The McFarland 2 and 24 h protocol differed in only one more sensitive result (trimethoprim). Comparing the McFarland 2 and 48 h incubation time protocol to the recommended one, there was one more resistant result (tylosin).

For strain 2 all four protocols produced exactly the same results and no differences were observed. The results were 14 x sensitive, 1 x intermediate and 8 x resistant.

With the recommended protocol strain 3 was sensitive to the tested substances 15 x, intermediate 1 x and resistant 7 x. Again, when looking at the same McFarland concentration but only a 24 h incubation time, there were two less resistant results. One turned into intermediate (nalidixic acid) and one was tested sensitive (trimethoprim). The McFarland 2 and

24 h protocol resulted in only one difference. Nalidixic acid was intermediate instead of resistant. No difference could be observed when comparing the results of the McFarland 2 and 48 h incubation time protocol with the recommended one.

The differences between the protocols were mainly less results classified as resistant for the antibiotics nalidixic acid and trimethoprim when shortening the incubation time from 48 to 24 hours. Nalidixic acid inhibits the DNA gyrase activity of bacteria and is bacteriostatic, hence hindering the growth of microbes, but does not kill them (Sugino et al. 1977).

Trimethoprim binds to the dihydrofolate reductase of bacteria and thus stops the reduction of dihydrofolic acid to tetrahydrofolic acid in the bacteria cell. Tetrahydrofolic acid plays an important role in thymidine synthesis pathway, therefore every interference with the production of tetrahydrofolic acid disrupts the bacterial DNA synthesis. Hence, trimethoprim has a bacteriostatic effect on bacteria. (Brogden et al. 1982)

Looking at the two mechanism of action it appears to be comprehensible, that a 24 h incubation time leads to the result sensitive, while a 48 h incubation time shows that the strain is resistant. Despite being able to grow under the presence of these antibiotic substances the bacterial growth of resistant strains might be slowed. When looking at slower growing bacteria like *O. rhinotracheale* there might not be enough bacterial growth in the wells to result in a measurable growth after just 24 h incubation time although significant bacterial growth was happening. This would lead to false sensitive results in the process of testing. After 48 h of incubation the Micronaut-S system is able to detect the growth better and therefore test results are more reliable. This shows the importance of a longer incubation time, when determining the resistance profile of *O. rhinotracheale*.

Regarding the protocols using a McFarland 2 bacterial suspension the same is true for the 24 h incubation time. Results were slightly better compared to McFarland 0.5 but still two resistant results were missed compared to the recommended McFarland 0.5 and 48 h incubation time (nalidixic acid and trimethoprim). When incubating the McFarland 2 variant for 48 h all results except for one were the same compared to the recommended protocol. Strain 1 was classified as resistant to tylosin with the McFarland 2 and 48 h protocol.

Due to the overall better recognition of resistant results when incubating for 48 h and no clear advantage of using a McFarland 2 bacterial suspension, the recommended protocol (McFarland 0.5 and 48 h incubation time) was applied within the whole study. Summarizing, the following method was chosen to conduct the antibiotic susceptibility testing on the collected

strains: 200 µl McFarland 0.5 bacteria suspension in Lysogeny broth with 2.5 % horse blood; incubation for 48 h under microaerophilic conditions at 37 °C; analysis with software setting H60 with MIC values according to CLSI standards. After applying this method to all *O. rhinotracheale* strains in this study it was evaluated for its suitability for antibiotic susceptibility testing with *O. rhinotracheale.*

4.3.2 Results of the evaluation of the identified antibiotic susceptibility test protocol for *O. rhinotracheale*

The aim of the present study was to identify and evaluate a method to use microdilution with Micronaut-S for consistent resistance testing of *O. rhinotracheale* strains. The method described in 4.3.1 was used for all 66 *O. rhinotracheale* strains. It was possible to measure valid MIC results for 65 of these 66 strains, one strain did not show any growth, neither in the positive control nor in the antibiotic testing wells. This means, that the used method was able to produce consistent growth and usable results for 98.48 % of strains. The rate of no or too little growth was 1.52 %. Additionally, for one strain Micronaut-S was not able to measure one out of the 23 MIC values, namely ampicillin. When using this method in practice, it would be possible to verify the result visually. In total 96.96 % of the strains were measured completely successful, 1.52 % would need additional visual examination due to minor uncertainties and 1.52 % of the strains did not grow (table 12).

Table 12 *Results of evaluation of susceptibility testing method*

	O. rhinotracheale strains included in study	strains which did not show sufficient growth	strains for which individual MIC values could not be achieved	strains which were tested successfully
Total number	66	1	1	64
Percentage	100 %	1.52 %	1.52 %	96.96 %

4.4 Antibiotic susceptibility testing

Over the last two decades studies about the resistance profiles of *O. rhinotracheale* strains have been published in different countries (van Veen et al. 2001; Szabó et al. 2015; Peña-Vargas et al. 2016; Malik et al. 2003; Churria et al. 2016). One major aim of this thesis was to assess the situation of antibiotic resistance in *O. rhinotracheale* strains isolated from Austrian turkey flocks.

The *O. rhinotracheale* strains included in this study were largely sensitive (98.4 %) to amoxicillin. Combining amoxicillin with clavulanic acid increased susceptibility to 100 %. Ampicillin was effective in 98.4 % of the strains. However, there is no registered drug containing ampicillin for poultry with this combination in Austria (Bundesamt für Sicherheit im Gesundheitswesen 2021). Another substance of the family of penicillins, oxacillin achieved a sensitive result for only 10.8 % of the strains.

Looking at the four tested antimicrobials out of the group of cephalosporins, consistent results were achieved. Against cefazolin, a cephalosporin of the first generation, 84.6 % of *O. rhinotracheale* strains were sensitive. Cefoxitin, a cephalosporin of the second generation hindered growth of 100 % of the strains. To cefotaxime, a cephalosporin of the third generation 87.7 % of the strains were susceptible. Ceftazidime, also a cephalosporin of the third generation was effective against 86.2 % of the strains.

Against imipenem belonging to the carbapeneme 98.4 % of the strains were sensitive.

All *O. rhinotracheale* strains in this study showed resistance against the three tested aminoglycosides, gentamicin, neomycin and streptomycin.

Interestingly, only 1.5 % of the strains were sensitive to enrofloxacin, a fluoroquinolone, while 89.2 % of strains were intermediate and 9.2 % were resistant. Against another fluoroquinolone, namely nalidixic acid, only 1.5 % of the strains were sensitive and 7.7 % intermediate. 90.8 % of strains proofed to be resistant against this antimicrobial.

All isolates were susceptible to chloramphenicol. Resistance against tetracycline were also low with 87.7 % of sensitive strains. In addition, 87.7 % of strains showed no resistance against tylosin. Resistance against colistin was found in all *O. rhinotracheale* strains.

Trimethoprim resistance was quite frequent with only 23.1 % sensitive results. All isolates were susceptible to sulfamethoxazole as well as to the combination of trimethoprim and sulfamethoxazole, also known as cotrimoxazole.

The results are summarized in table 13.

Table 13 *Percentages of "sensitive", "intermediate" and "resistant" results of O. rhinotracheale strains from 2002 to 2021 for each tested antibiotic substance*

Antibiotic substance	Sensitive results in %	Intermediate results in %	Resistant results in %
Amoxicillin	98.4 %	0.0 %	1.5 %
Amoxicillin / clavulanic acid	100.0 %	0.0 %	0.0 %
Ampicillin	98.4%	0.0 %	1.5 %
Oxacillin	10.8%	0.0 %	89.2 %
Cefazolin	84.6%	1.5 %	13.8 %
Cefoxitin	100.0%	0.0 %	0.0 %
Cefotaxime	87.7%	0.0 %	12.3 %
Ceftazidime	86.2%	0.0 %	13.8 %
Imipenem	98.4%	1.5 %	0.0 %
Gentamicin	0.0%	0.0 %	100.0 %
Neomycin	0.0%	0.0 %	100.0 %
Streptomycin	0.0%	0.0 %	100.0 %
Enrofloxacin	1.5%	89.2 %	9.2 %
Nalidixic acid	1.5%	7.7 %	90.8 %
Chloramphenicol	100%	0.0 %	0.0 %
Tetracycline	87.7%	10.8 %	1.5 %
Tylosin	87.7%	0.0 %	12.3 %
Colistin	0.0%	0.0 %	100.0 %
Trimethoprim	23.1%	0.0 %	76.9 %
Sulfamethoxazole	100.0%	0.0 %	0.0 %
Trimethoprim / Sulfamethoxazole	100.0%	0.0 %	0.0 %
Trimethoprim / Sulfamethoxazole urinary infection	100.0%	0.0 %	0.0 %
Trimethoprim / Sulfamethoxazole systemic infection	13.8%	0.0 %	86.2 %

In order to assess the general development of resistance of *O. rhinotracheale* strains against antibiotic substances over time, the sums of “sensitive”, “intermediate” and “resistant” results were calculated in percent/year (figure 14). With this it is possible to compare the ratio of “sensitive”, “intermediate” and “resistant” results for all antibiotics and isolates of every year. In most years about 60 % of the results are sensitive with 54 % and 68 % as lowest and highest percentage respectively and one outlier (2005) with over 90 % sensitive results. In 2005 only one strain was isolated and examined, therefore results should not be overestimated.

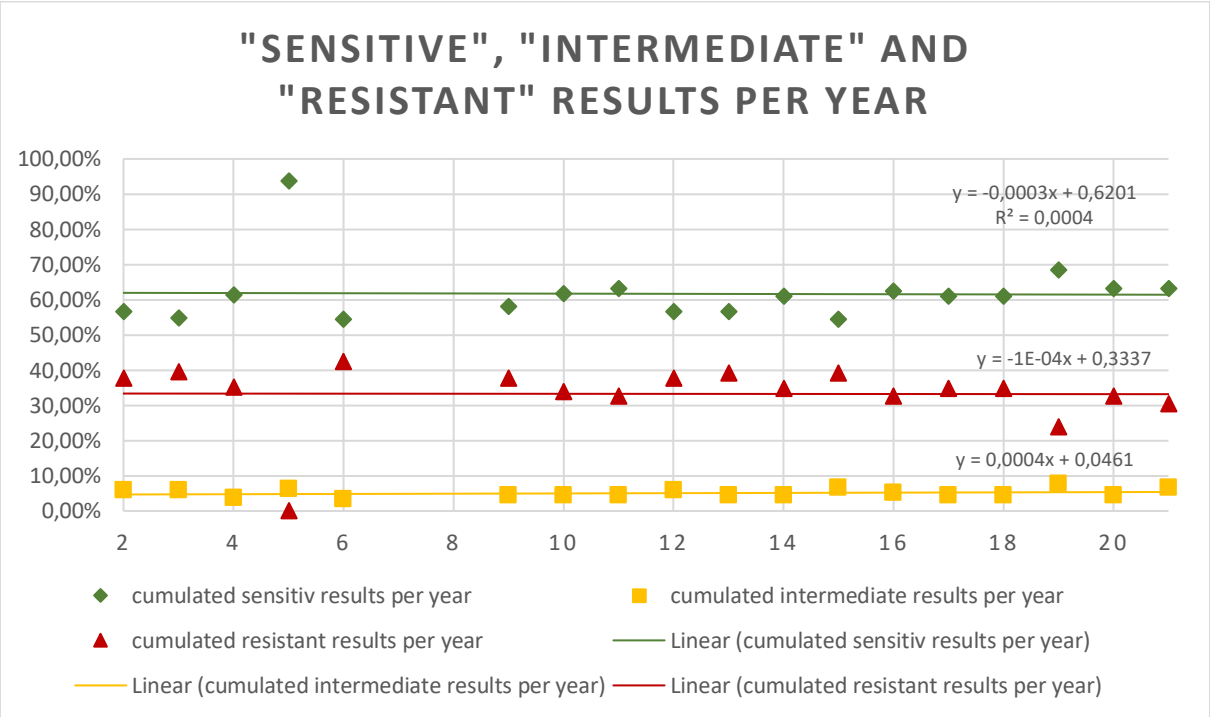


Figure 14 *Results of antibiotic susceptibility testing cumulated for all isolates and antibiotic substances per year*

The linear trend line shows that the percentage of sensitive results hardly changed over the last two decades. This, of course, means that also the overall percentage of resistance of *O. rhinotracheale* strains against antibiotic substances did not change significantly either. This is in contrast to previous studies, which reported rising resistance rates from different classes of antibiotics (van Veen et al. 2001; Malik et al. 2003; Churria et al. 2016; Peña-Vargas et al. 2016).

In addition to the overall development of resistance also the trend of susceptible and not susceptible results of each antibiotic substance between 2002 and 2021 was analysed (figures 15-26).

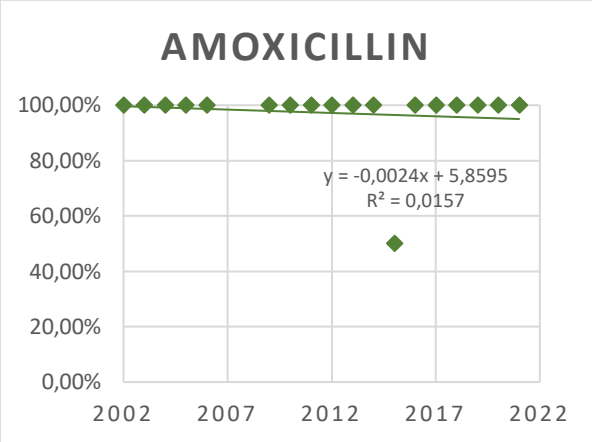
As in the results for all years combined, also in the analysis per year the high rate of sensitive isolates against aminopenicillins is visible. Amoxicillin resistance occurred only in one year, when 50 % of strains were not susceptible to the antibiotic. In all other years all strains included in this study were sensitive to amoxicillin.

This is in contrast to data from the Netherlands, where only 36,9% of strains were susceptible to amoxicillin and resistance has become more frequent over the investigated time (van Veen et al. 2001). Strains from Malaysia showed 100 % resistances and a recent study from Hungary reported 40 % of isolates as resistant (Mohd-Zain et al. 2008; Szabó et al. 2015). Results from two studies from Taiwan with strains isolated from chickens and from India with strains from laying hens were in agreement with the present findings proving high susceptibility of *O. rhinotracheale* to amoxicillin (Tsai and Huang 2006; Murthy et al. 2008). The near absence of resistance against amoxicillin in Austria is surprising, because amoxicillin is a first line antimicrobial registered for treatment of O. rhinotracheale infections (Bundesamt für Sicherheit im Gesundheitswesen 2021).

Ampicillin produced exactly the same results as amoxicillin. This finding is in accordance with results from Taiwan and India with 96.4 % and 100 % sensitive results respectively (Tsai and Huang 2006; Murthy et al. 2008). However, it is in contrast to previous reports from US, Argentina, Hungary, Malaysia and Mexico, in which strains were found resistant to this antimicrobial (Malik et al. 2003; Mohd-Zain et al. 2008; Szabó et al. 2015; Churria et al. 2016; Peña-Vargas et al. 2016).

All strains were susceptible to the combination of amoxicillin and clavulanic acid. The addition of clavulanic acid caused less resistant results in a study from Mexico too, but still around 13% of strains were resistant (Peña-Vargas et al. 2016).

Resistance against oxacillin, which belongs to another sub-group of penicillins, seem to be far more widespread in Austria compared to the aminopenicillins. Furthermore, the rate of resistance rose over the years. In most recent years nearly all strains proved to be resistant against oxacillin.



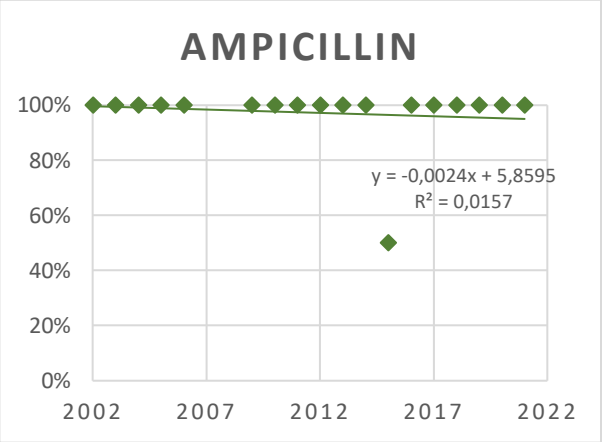
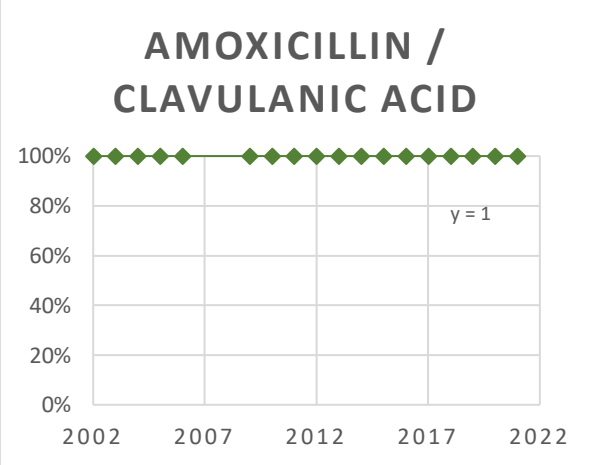


Figure 15 a and b *Susceptibility of O. rhinotracheale strains to amoxicillin (a) and ampicillin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*



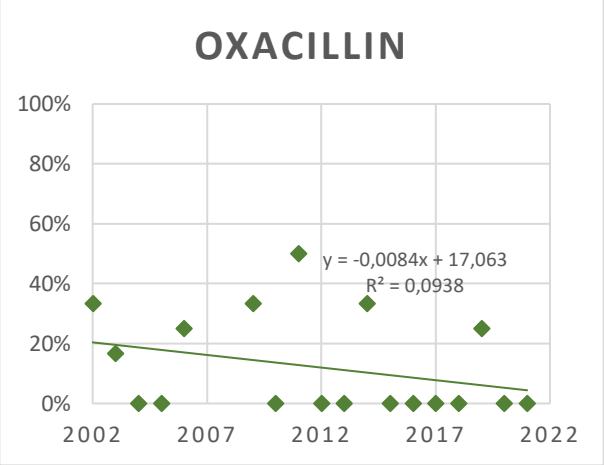


Figure 16 a and b *Susceptibility of O. rhinotracheale strains to amoxicillin / clavulanic acid (a) and oxacillin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

Resistance against the tested cephalosporins were rare. All *O. rhinotracheale* strains in this study were susceptible to cefoxitin. A look at the development of resistance against the other three cephalosporins between 2002 and 2021, reveals a distinct trend towards less resistance. Whereas in the first half of the time span often only about 70 % of the isolates of one year were susceptible to cefazolin, cefotaxime or ceftazidime, in the last years no resistance occurred at all. Cefazolin showed a slightly different result compared to the others because of a 50 % resistance rate in 2015, but since then all isolates seem to be susceptible to cefazolin as well.

The low rates of resistance and the trend towards higher susceptibility are in contrast to findings from the Netherlands, the United States, India and Hungary, where resistant strains are remarkably more frequent (van Veen et al. 2001; Malik et al. 2003; Murthy et al. 2008; Szabó et al. 2015). A study from Taiwan (Tsai and Huang 2006) had similar results as the present study. In all other studies less cephalosporins were tested in comparison to this study and the main cephalosporin was ceftiofur, which belongs to the 3rd generation and was not used in the present study (van Veen et al. 2001; Malik et al. 2003; Tsai and Huang 2006; Szabó et al. 2015). In contrast to other countries (van Veen et al. 2001), cephalosporins are generally not used in poultry in Austria (Bundesamt für Sicherheit im Gesundheitswesen 2021).

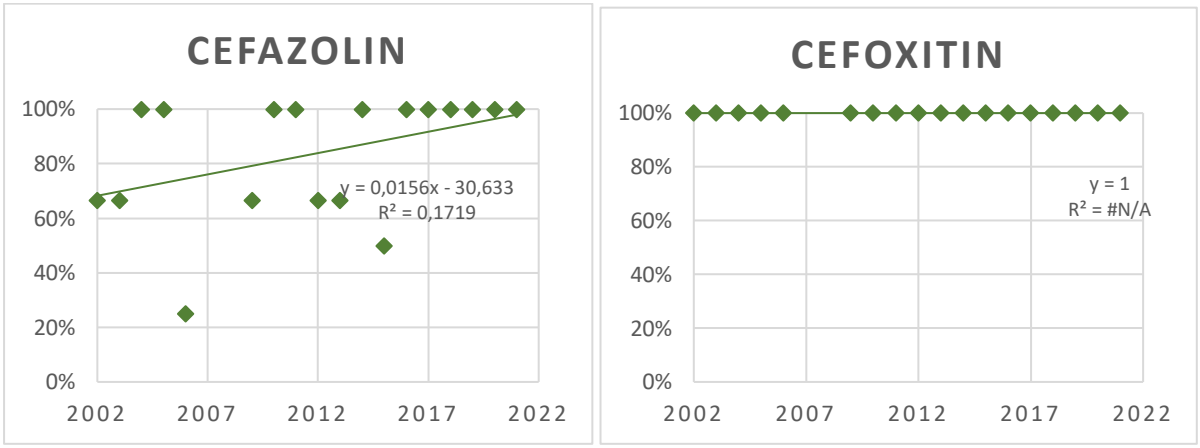


Figure 17 a and b *Susceptibility of O. rhinotracheale strains to cefazolin (a) and cefoxitin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

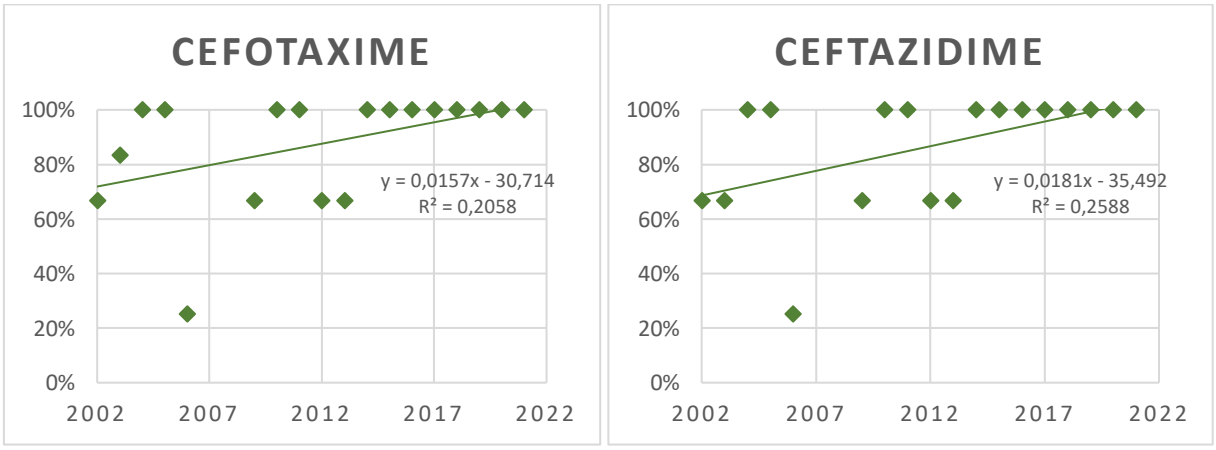


Figure 18 a and b *Susceptibility of O. rhinotracheale strains to cefotaxime (a) and ceftazidime (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

The last antibiotic out of the β-lactam-antibiotics that was included in this study was imipenem, a carbapenem. In all but one year all *O. rhinotracheale* strains were susceptible to imipenem. In 2017 one third of the isolates was resistant against the antibiotic. Imipenem is not registered for the use in poultry (Bundesamt für Sicherheit im Gesundheitswesen 2021).

All strains in this study showed resistance against aminoglycosides. It was not possible to determine any trend for gentamicin, neomycin and streptomycin, since none of the strains was susceptible. This is in accordance with findings from many countries (van Veen et al. 2001; Ak and Turan 2001; Murthy et al. 2008; Szabó et al. 2015; Churria et al. 2016; Peña-Vargas et al. 2016). An investigation of strains from the USA isolated between 1996 and 2002 found up to 40 % of strains from the first years susceptible to gentamicin, however, 100 % of strains were resistant in the upcoming years (Malik et al. 2003). Similarly, in Taiwan resistance to gentamicin were not 100 %, but still high with 75 % (Tsai and Huang 2006). Due to the high rate of resistance to aminoglycosides, sometimes the usage of agar with gentamicin as additive is recommended for *O. rhinotracheale* cultivation (Swayne 2020). Looking at the possibility of susceptible strains, as seen in the USA and Taiwan, this might lead to false negative results.

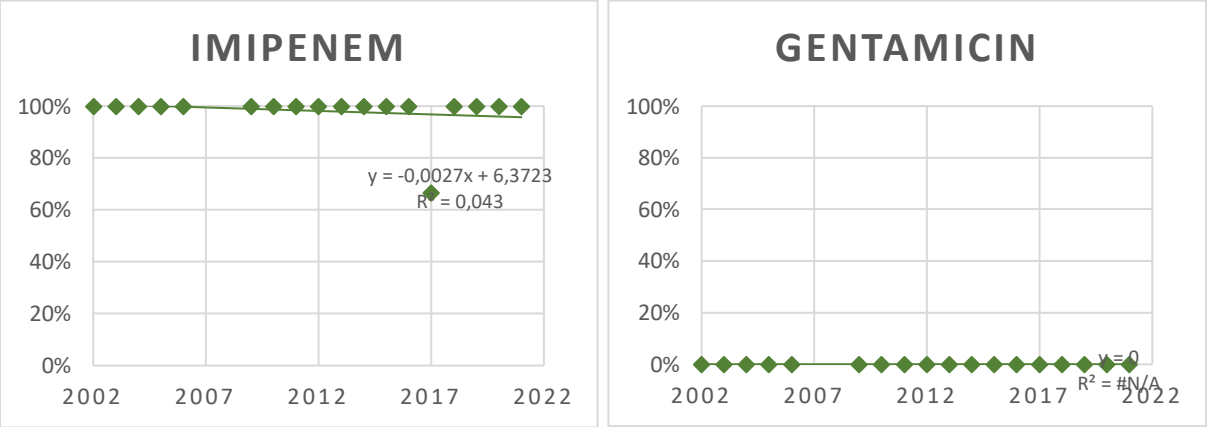


Figure 19 a and b *Susceptibility of O. rhinotracheale strains to imipenem (a) and gentamicin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

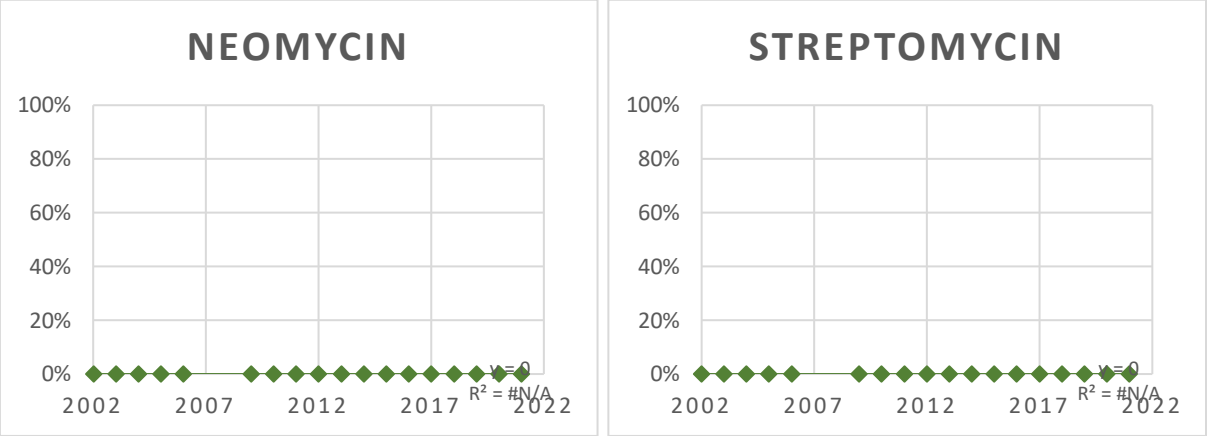


Figure 20 a and b *Susceptibility of O. rhinotracheale strains to neomycin (a) and streptomycin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

Except one year (2013) there was no sensitive strain against Enrofloxacin. In 2013 33 % of the isolates were sensitive to this antimicrobial. Exactly the same results were achieved for nalidixic acid, a quinolone. No noticeable change of resistance over the years could be determined for both antibiotics. The rising trend curves in figure 21a and b are due to the one year with sensitive isolates. Interestingly, 89.2 % of the isolates are classified as intermediate susceptible to enrofloxacin. Only 9.2 % of the strains show an enrofloxacin resistance. Such a high percentage of intermediate results is unique to enrofloxacin. For nalidixic acid the percentage of intermediate results was a lot lower at 7.7 %. The number of nalidixic acid resistance is accordingly higher and amounts to 90.8 %.

Very low rates of sensitive results to enrofloxacin were also reported from the Netherlands, Malaysia, Hungary and Argentina (van Veen et al. 2001; Mohd-Zain et al. 2008; Szabó et al. 2015; Churria et al. 2016). Some authors found about 50 % of strains to be resistant to enrofloxacin, but did not publish numbers for intermediate and sensitive results (Malik et al. 2003; Tsai and Huang 2006). Only in a study from India 100 % of strains are reported to be susceptible to enrofloxacin.

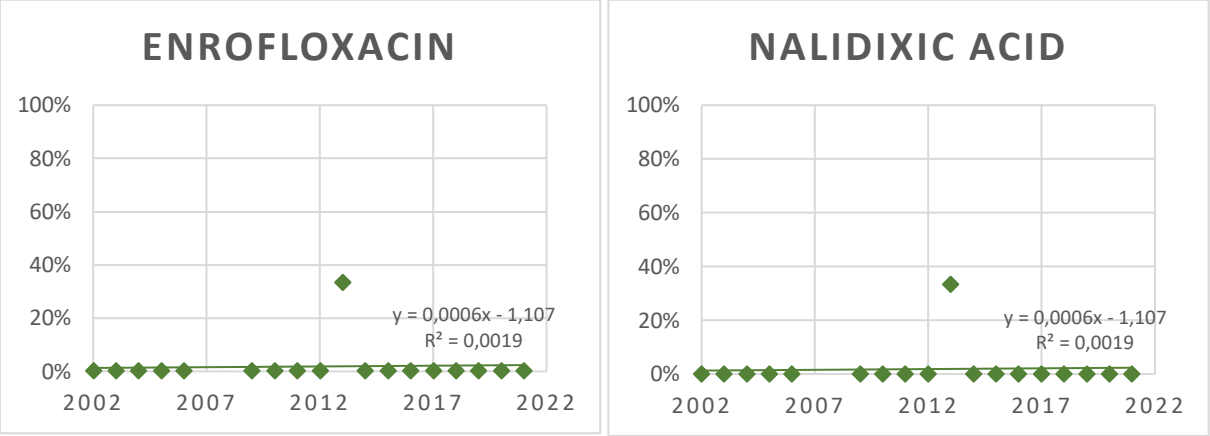


Figure 21 a and b *Susceptibility of O. rhinotracheale strains to enrofloxacin (a) and nalidixic acid (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

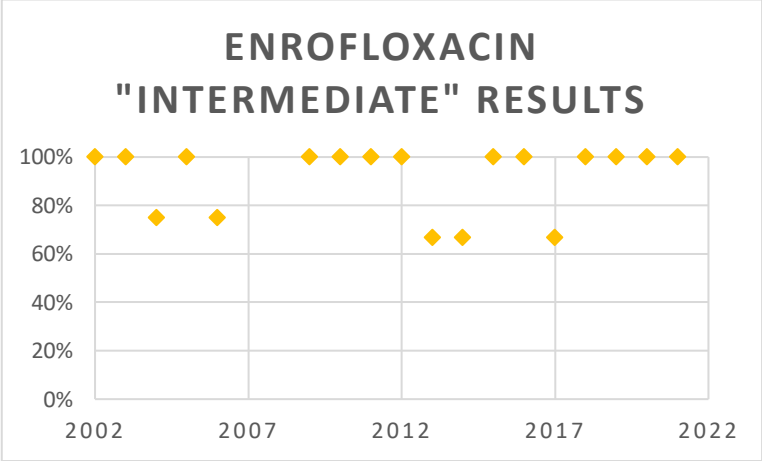


Figure 22 *Graph of intermediate results of O. rhinotracheale strains to enrofloxacin from 2002 to 2021. The graph shows intermediate results (%) / year.*

Chloramphenicol, an antibiotic substance, strictly forbidden for veterinary use in farm animals in the European Union (European Commission 2009) was able to produce sensitive results for 100 % of the *O. rhinotracheale* strains. High sensitivity rates were expected due to the total ban in farm animals. Results for chloramphenicol are in agreement with findings from India, Malaysia and Hungary (Murthy et al. 2008; Mohd-Zain et al. 2008; Szabó et al. 2015).

Strains resistant to tetracycline were isolated in several years. The overall rate of resistance was rather low and in many years 100 % of strains were susceptible. Also, the trend curve indicates an increase in sensitive results, hence a decrease in tetracycline resistance.

These results are in contrast to findings from The Netherlands, Taiwan and Mexico, where most strains were found resistant to tetracycline (van Veen et al. 2001; Tsai and Huang 2006; Peña-Vargas et al. 2016). The low rate of resistance is in agreement with the data from Malik et al. (2003), but they found a rising resistance to tetracycline.(Malik et al. 2003)

Resistance against tylosin were also very rare. In most years all isolated *O. rhinotracheale* strains were susceptible to this antimicrobial. Furthermore, the trend curve shows a weak increase in susceptible strains between 2002 and 2021. In contrast to the present study data from The Netherlands and Malaysia showed very high MIC values (van Veen et al. 2001; Mohd-Zain et al. 2008).

Colistin resistance seems to be a common feature of *O. rhinotracheale*, since 100 % of the isolates were resistant to it. Therefore, also no change over time can be described. Colistin is very rarely used for antibiotic susceptibility testing for *O. rhinotracheale*, however, the 100 % resistant results in the present study are in agreement with findings from Mexico (Peña-Vargas et al. 2016).

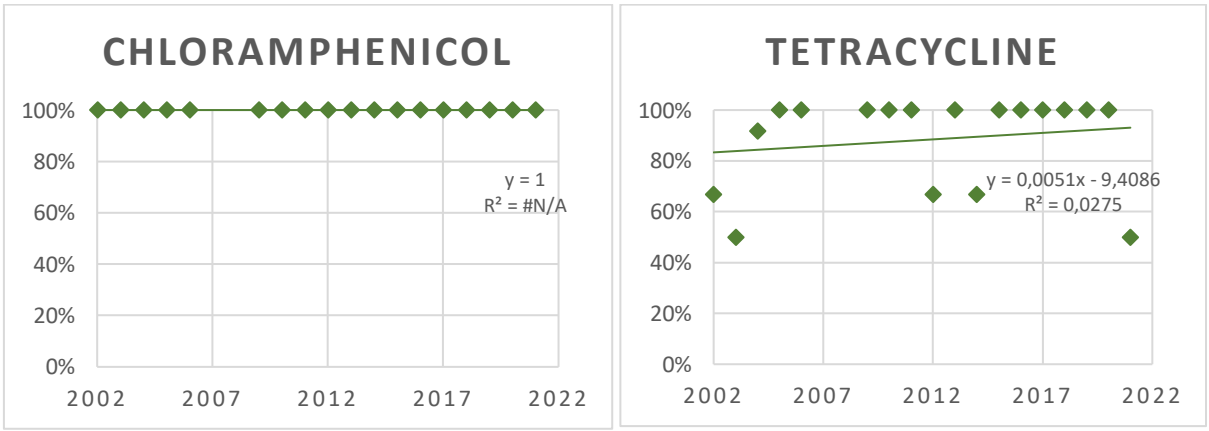


Figure 23 a and b *Susceptibility of O. rhinotracheale strains to chloramphenicol (a) and tetracycline (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

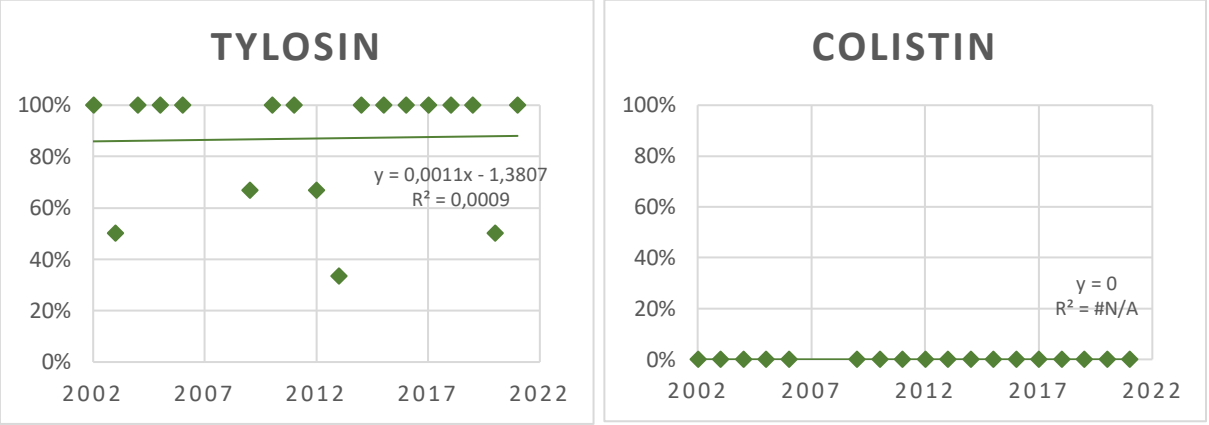


Figure 24 a and b *Susceptibility of O. rhinotracheale strains to tylosin (a) and colistin (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

O. rhinotracheale strains resistant to trimethoprim were quite common. In most of the years none to only a third of the isolates were susceptible. Only in three years more than 70 % of strains were susceptible to trimethoprim. The trend curve indicates an increase of susceptible results over the years. Sulfamethoxazole proved to be very effective. All *O. rhinotracheale* strains in this study proved susceptible. Trimethoprim and sulfamethoxazole on their own are rarely used for susceptibility testing for *O. rhinotracheale*, most authors chose the combination of the two antibiotics for susceptibility testing (van Veen et al. 2001; Malik et al. 2003; Tsai and Huang 2006; Szabó et al. 2015; Churria et al. 2016; Peña-Vargas et al. 2016).

The combination of trimethoprim and sulfamethoxazole uses a well-known synergy and is rather common (Brian J. Werth 2020). This finding is in clear contrast to findings from the Netherlands, the United States of America, Taiwan, Hungary, Argentina and Mexico, where most isolates proved resistant (van Veen et al. 2001; Malik et al. 2003; Tsai and Huang 2006; Szabó et al. 2015; Churria et al. 2016; Peña-Vargas et al. 2016).

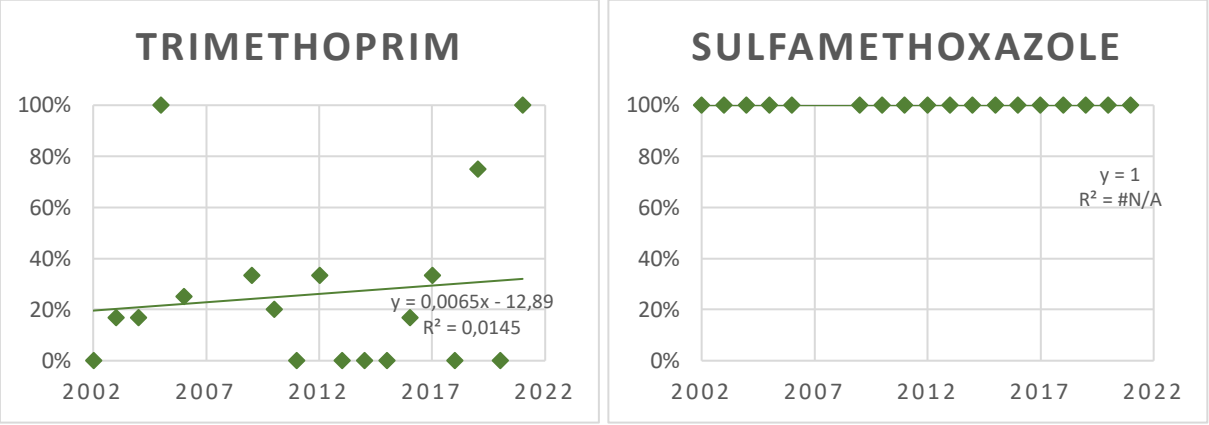


Figure 25 a and b *Susceptibility of O. rhinotracheale strains to trimethoprim (a) and sulfamethoxazole (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

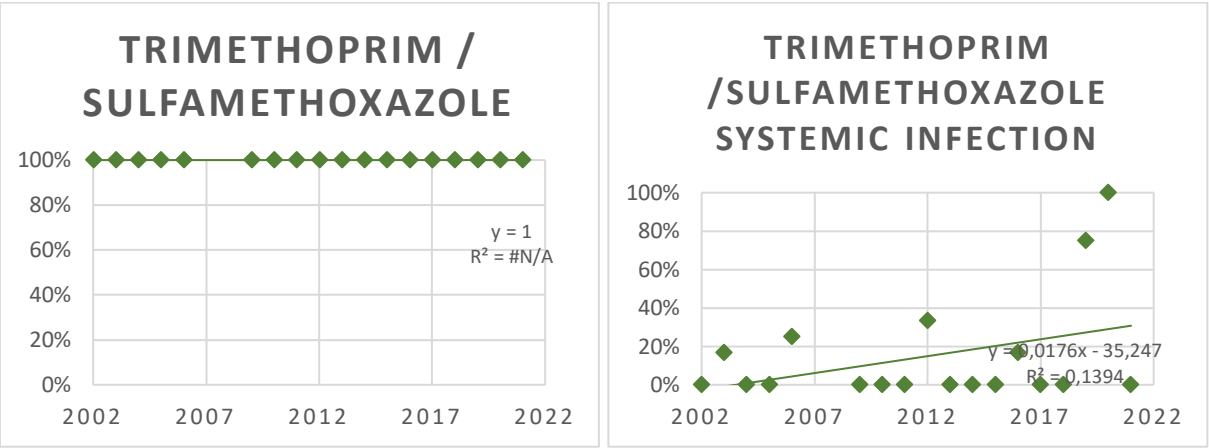


Figure 26 a and b *Susceptibility of O. rhinotracheale strains to trimethoprim / sulfamethoxazole (a) and trimethoprim / sulfamethoxazole systemic infection (b) from 2002 to 2021. Each graph shows susceptibility (%) / year and the corresponding linear trend curve.*

4.4.1 Correlation of phenotypic properties and antibiotic susceptibility

Because of the big variety of phenotypes of *O. rhinotracheale* isolates and their rather different antibiotic resistance profiles, the question occurred, whether there is a correlation between phenotype and antibiotic resistance. To gain some insights the strains included in this study were grouped according to certain phenotypic properties and correlated with their antibiotic resistance profiles. Literature on the effects of the phenotype is generally limited. In the United States the pathogenicity of *O. rhinotracheale* isolates depending on β-hemolysis was analysed

(Walters et al. 2014). A group from Argentina compared antibiotic susceptibility of hemolytic and nonhemolytic *O. rhinotracheal*e strains (Churria et al. 2016). In China it was shown that small colony variants of *O. rhinotracheale* tended to be more resistant to antibiotic substances (Zahra et al. 2013).

To obtain a very simple comparison of antimicrobial resistance the overall percentage of sensitive results was calculated for each group (table 14). The reference value of all *O. rhinotracheale* strains in this study is 60.26 %. This was achieved by summing up all results from all isolates, with around 60 % of the results being sensitive. Of course, all antibiotics which face 100 % resistance (gentamicin, neomycin, streptomycin and colistin) also get 100 % resistant results in the phenotypic property subgroups. Therefore, these antibiotics will be excluded from this type of analysis.

Table 14 *Percentages of "sensitive" results of O. rhinotracheale strains with different phenotypic features for selected antibiotic substances*

	susceptibility (%)			
antimicrobial substance	all strains	strains with β-hemolysis	strains with colonies < 0.7 mm	strains with colonies < 1.2 mm
overall	60.26 %	60.00 %	58.89 %	58.39 %
amoxicillin	98.4 %	90.00 %	100.00 %	97.62 %
ampicillin	98.4 %	88.89 %	100.00 %	97.56 %
cefazolin	84.6 %	80.00 %	77.27 %	76.19 %
cefoxitin	100.00 %	100.00 %	100.00 %	100.00 %
cefotaxime	87.7 %	90.00 %	77.27 %	80.95 %
ceftazidime	86.2 %	90.00 %	77.27 %	78.57 %
enrofloxacin	1.5%	0.00 %	4.55 %	2.38 %
tetracycline	87.7 %	80.00 %	86.36 %	83.33 %
tylosin	87.7 %	90.00 %	86.36 %	83.33 %
trimethoprim	23.1 %	60.00 %	13.64 %	21.43 %

The overall percentage of sensitive results for strains capable of β-hemolysis was 60.00 %, and no differences in resistance profiles were found. This is in accordance with results from

Argentina, where no statistically significant difference between strains with and without β-hemolysis was found (Churria et al. 2016). However, when looking at the individual antibiotic substances, some differences were noticeable (table 14). It is important to keep in mind, that the number of strains with β-hemolysis is very limited (n=10) and therefore a very small number of strains with different results cause a high relative difference. Interestingly, the investigation of strains from Argentina also found some differences, when looking at single antibiotics, although in that study the number of strains was low as well and therefore the significance of differences is small (Churria et al. 2016).

Aminopenicillins without clavulanic acid may be less effective against β-haemolytic strains (90 % to 98.5 % for amoxicillin and 88.9 % to 98,4 % for ampicillin). Resistance against cephalosporins seem to be approximately the same.

No difference was found in the susceptibility rate against the fluoroquinolone enrofloxacin. The strains with β-hemolysis showed slightly more resistance against tetracycline (80 % to 87.7 %) while results for tylosin were quite similar. The β-haemolytic strains were noticeably more susceptible to trimethoprim (60 % to 23.1 %). When interpreting these results, it is important to keep the low number of strains with β-hemolysis in mind.

To compare antibiotic susceptibility of small colony variant strains, all isolates with colonies smaller than 0,7mm (the two smallest groups in the size classification described above) were analysed (table 14). The overall percentage of sensitive results was 58.89 %. This is slightly less than the 60.26 % of all strains included in the study. These findings are in accordance with results for O. rhinotracheale SCVs (Zahra et al. 2013) and the supposedly higher resistance rates for SCVs in general (Proctor et al. 1998; Proctor et al. 2006; Melter and Radojevič 2010). Isolates with very small colonies did not show any resistance against aminopenicillins. Therefore, results for amoxicillin and ampicillin are similar to the whole group. This contradicts the findings from China (Zahra et al. 2013) and the general tendency that SCVs are less susceptible to β-lactam antibiotics (Melter and Radojevič 2010).

Resistance against cephalosporins seem to be more frequent in isolates with very small colonies. All strains were only sensitive to cefoxitin, a 2nd generation cephalosporin. All other three tested antibiotics of the cephalosporins were less effective. 77.27 % of strains were sensitive to cefazolin compared to 84.60 % of all strains. Furthermore, 77.27 % of isolates were susceptible to cefotaxime and ceftazidime compared to 87.70 % and 86.20 %

respectively. With these results the lower susceptibility to β-lactam antibiotics (Melter and Radojevič 2010) could be confirmed for such strains in the present study.

4.55 % of small colony strains were susceptible to enrofloxacin while only 1.50 % of all strains were susceptible. This difference is caused by only one strain. Therefore, the importance should not be overestimated. Resistance against tetracycline and tylosin were approximately the same. Trimethoprim resistance were more frequent in small colony isolates with 13.64 % of sensitive strains compared to 23.10 % in all strains.

When including strains with colonies between 0.7 mm and 1.2 mm, hence assessing all isolates with colonies under 1.2 mm, the overall percentage barely changed to 58.39 % (table 14). This could mean that the characteristics of SCVs that lead to a slightly lower susceptibility also extend to isolates with colonies of around 1 mm. Resistance against cephalosporins were still more frequent than in all strains. Differences of relative susceptible results for enrofloxacin got smaller due to the higher number of strains included.

Tetracycline and tylosin resistance were more frequent after including strains with colonies between 0.7 mm and 1.2 mm. Strains were susceptible to both antibiotics in 83.33 % compared to 86.36 % before and 87.70 % in all strains. On the other side with 21.43 % more strains were susceptible to trimethoprim compared to 13.64 % without strains with colonies between 0.7 mm and 1.2 mm.

The results of both comparisons show that the size of colonies may have an impact on the susceptibility to antibiotics. Antibiotic resistance occur possibly more frequent in isolates with small colonies, as already discussed by Zahra et al. (2013).

Almost 15 % of the strains showed an extraordinarily high heterogeneity in their appearance, but did not reveal a difference in antibiotic sensitivity compared to other strains (60.87 % to 60.26 %). On the level of individual antibiotic substances a few differences may exist. 11.11 % of strains were susceptible to enrofloxacin compared to only 1.50 % of all strains. The big percentage difference is caused by the small number of strains with high heterogeneity and actually due to only one isolate.

All strains with high heterogeneity were susceptible to tetracycline, in comparison to 87.70 % of all strains. In contrast, trimethoprim resistance was more frequent with 11.11 % of strains susceptible compared to 23.10 %. Again, the difference in percent may be magnified by the small number of isolates.

Approximately 20 % of *O. rhinotracheale* strains had a light reddish colour also described as reddish glow (van Empel et al. 1997) and therefore differed somehow strongly from the classical appearance of *O. rhinotracheale* colonies. However, their overall sensitivity (%) with 60.14 % was nearly the same as 60.20 % for all strains.

On an individual antibiotic substance level some differences appeared, although the magnitude of the percentage differences may be affected by the quite small number of strains. Aminopenicillin resistance may be more relevant for reddish colony strains with 91.67 % susceptible isolates comparing to 98.50 % in all strains for both amoxycillin and ampicillin. However, these differences are caused by only one strain.

In contrast, these strains seem to be more susceptible to cephalosporins with 91.67 %, 100 % and 100 % of strains sensitive to cefazolin, cefoxitin and ceftazidime respectively. Also, more isolates were susceptible to tylosin with 100 % compared to 87.70 %.

Strains with more transparent colonies had a lower sensitivity with 57.97 % compared to 60.2 %. Resistance against cephalosporins was more frequent with only 73.33 % of strains being susceptible to cefazolin, cefotaxime and ceftazidime, respectively, compared to 84.6 % for cefazolin, 87.7 % for cefotaxime and 86.2 % for ceftazidime. With 6.67 % of strains susceptible to enrofloxacin there was quite a difference to the 1.5 % of all strains, though the difference was caused by only one strain.

Resistances to tetracycline and especially tylosin occurred more frequently compared to all strains. Strains with transparent colonies were susceptible to tetracycline in 73.33 % compared to 87.70 % of all isolates. Only 66.67 % of the more transparent isolates were sensitive to tylosin while 87.70 % of all strains were susceptible. In contrast, more strains were susceptible to trimethoprim with 33.33 % compared to 23.10 % of all strains.

These results might suggest an influence of the described differences in phenotypic characteristics of *O. rhinotracheale* on the antibiotic susceptibility, especially for cephalosporins, tetracyclines and macrolide antibiotics. Another possible explanation are mutual underlying mechanisms, that influence phenotype and antibiotic susceptibility.

4.4.2 Repeated outbreaks on farms and their effect on antibiotic susceptibility

20 strains were isolated from nine farms with multiple outbreaks within one year. *O. rhinotracheale* strains isolated from these farms were analysed on a farm level to explore any

correlation between repeated occurrence of *O. rhinotracheale* and its susceptibility to antibiotics (table 15). Whether any antibiotic treatment was carried out in these farms is not known.

The number of sensitivity changes was counted for each repeated outbreak. This includes changes towards more resistance, towards more sensitivity and the combination of both (more resistance minus more sensitive results). A change from sensitive to intermediate, intermediate to resistant, resistant to intermediate and intermediate to sensitive was also counted as a change of susceptibility.

Out of the 9 farms with multiple outbreaks in 7 of them a change of susceptibility to at least one antimicrobial occurred. Only in 2 farms the resistance profile of the isolated strains stayed exactly the same. In 4 of the 7 farms with susceptibility changes the strains of the repeated outbreaks were more resistant than the strains of the first outbreak. In 2 of the 7 farms the isolates of the repeated outbreaks were less resistant compared to the isolates of the first outbreak. In 1 of the 7 farms the resistance profile changed, but the total number of resistance stayed the same.

This analysis indicates, that *O. rhinotracheale* is very likely to gain and loose resistance mechanisms, in order to adapt to its environmental conditions. This ability to easily change its resistance profile is also described in literature (van Veen et al. 2001; Swayne 2020).

The antibiotics with the most changes of susceptibility, including changes from susceptible/intermediate to resistant and from resistant to susceptible/intermediate, were trimethoprim with four and enrofloxacin and oxacillin with three changes (table 16). Resistance against nalidixic acid changed two times. For tylosin one change of susceptivity was measured. That *O. rhinotracheale* is able to acquire resistance against these antibiotics, especially enrofloxacin, trimethoprim, tylosin and tetracyclin, was reported previously (van Veen et al. 2001; Malik et al. 2003; Szabó et al. 2015; Swayne 2020).

The number of differences in the resistance profile, hence the sum of every different result for each antibiotic between two strains from the same farm, varied from 1 to 4. This shows the variability of the resistance profile of *O. rhinotracheale*.

Table 15 *change of resistance in farms with multiple outbreaks within one year*

farm number	number of outbreaks within one year	occurrence of changes in resistance profile	difference of number of resistance
4	2	no	0
8	2	yes	+1
9	3	yes	-1
11	2	yes	-1
19	3	yes	+1
24	2	yes	+1
32	2	yes	+1
34	2	no	0
35	2	yes	0

Table 16 *number of susceptibility changes per antimicrobial in farms with multiple outbreaks in one year*

antimicrobial	Total number of susceptibility changes	changes from susceptible or intermediate to resistant	changes from resistant to susceptible or intermediate
trimethoprim	4	2	2
enrofloxacin	3	2	1
oxacillin	3	2	1
nalidixic acid	2	0	2
tylosin	1	1	0

Repeated outbreaks during the investigated time period but not within one year occurred in 10 farms. The number of strains isolated in these farms over the years differs from 2 to 5. In 1 of the 10 farms the resistance profiles of the isolated strains were similar. In all other 9 farms resistance profiles changed.

When looking at the first and the last isolated strain from the same farm, 4 of the 9 farms with changes faced an increase in resistance (table 17). In the other 5 farms the number of resistance was reduced over time. The high number of changes of the resistance profile was

expected as known from previous studies, but that strains, which had lost resistance, outnumbered strains, which had gained resistance was unexpected (van Veen et al. 2001; Malik et al. 2003; Peña-Vargas et al. 2016; Swayne 2020).

19 strains were isolated in repeated outbreaks, hence as 2nd, 3rd, 4th or 5th strain from the same farm. When comparing each of those strains with the preceding one, 16 of the 19 strains showed a different resistance profile. Of these 16 strains 7 had developed a higher number of resistance while also 7 had less resistances than their predecessor. The remaining 2 strains had different resistance pattern, but the total number did not differ from the other strains.

The maximum difference of the total number of resistance between the first strain of an outbreak and the last was 5. The maximum number of changes between any two strains from the same farm was 8. These high numbers are remarkable, meaning that besides from some certain resistance like colistin and gentamicin and some always effective but forbidden antibiotics like chloramphenicol and Imipenem, *O. rhinotracheale* might be able to acquire and loose resistance mechanisms, as previously described (Malik et al. 2003).

The antibiotic with the highest number of changes of resistance was trimethoprim with 8 changes (table 18). The second most changes of susceptibility were found for tylosin with 5 changes. Resistance against cefazolin, ceftazidime, nalidixic acid and enrofloxacin changed 4 times. The susceptibility to cefotaxime changed in 3 farms. Oxacillin resistance changed two times. Only one change of susceptibility occurred for amoxicillin, ampicillin and tetracyclin. Changes of resistance against most of these antibiotic substances are well described in literature (van Veen et al. 2001; Malik et al. 2003; Szabó et al. 2015; Swayne 2020).

Table 17 *change of resistance in farms with multiple outbreaks over the years*

farm number	number of outbreaks over the years	occurrence of changes in resistance profile	difference of number of resistance
1	3	yes	-4
2	2	yes	+2
11	5	yes	-2
18	4	yes	+5
21	2	no	0
24	4	yes	+1
26	2	yes	-3
27	3	yes	-2
31	2	yes	+2
33	2	yes	-1

Table 18 *number of susceptibility changes per antimicrobial in farms with multiple outbreaks over the years*

antimicrobial	Total number of susceptibility changes	changes from susceptible or intermediate to resistant	changes from resistant to susceptible or intermediate
trimethoprim	8	4	4
tylosin	5	2	3
cefazolin	4	1	3
ceftazidime	4	1	3
nalidixic acid	4	2	2
enrofloxacin	4	2	2
cefotaxime	3	0	3
oxacillin	2	1	1
amoxicillin	1	1	0
ampicillin	1	1	0
tetracycline	1	1	0

4.4.3 Influence of the geographic region on the antibiotic susceptibility

Nearly all of the strains included in this study have their origin in three different areas of Austria. With 39 isolates the most frequent origin is Lower Austria. 15 strains were isolated in Upper Austria and 8 strains in Burgenland.

The overall percentage of sensitive results for all strains was 60.26 %. The percentage of sensitive results for the strains from Burgenland was lower with 55.98 %, which might be due to the low number of strains (n=8). In Lower Austria 61.31 % of the results were sensitive. In Upper Austria the percentage of sensitive results was 58,15 %.

Looking at the individual antibiotics some more differences are noticeable. Amoxicillin resistance did not occur in Burgenland and Lower Austria with sensitivity rates of 100 %. In Upper Austria only 93,75 % of the isolates were susceptible to amoxicillin. Resistance against cephalosporins was more common in Burgenland than in Lower Austria and Upper Austria. All isolates were susceptible to cefoxitin in all areas. 62.5 % of the isolates from Burgenland were sensitive against cefazolin, cefotaxime and ceftazidime compared to 84.60 %, 87.70 % and 86.20 % for whole Austria, respectively. In Lower Austria resistance was less frequent with sensitivity rates of 89.74 %, 92.31 % and 89.74 % for cefazolin, cefotaxime and ceftazidime, respectively. In Upper Austria the results were quite similar to the results of the whole study.

Strains susceptible to enrofloxacin were very rare in all three areas. Tetracycline resistance was more common in Burgenland and Upper Austria than in Lower Austria with 75 % and 81.25 % compared to 87.7 %. Strains from Lower Austria were more susceptible to tetracycline with 92.31 % of sensitive strains. There was no resistance against tylosin in Burgenland, while in Upper Austria resistance was quite frequent with only 75 % of sensitive results. Lower Austria had 92.31 % susceptible strains. The overall average was 87.7 %. Trimethoprim resistance was very common in all strains with 23.1 % of susceptible strains. In Burgenland only 12.5 % of strains were susceptible. Lower and Upper Austria did not differ much from the results of the whole study.

5 Summary

5.1 Summary

O. rhinotracheale is an important pathogen in poultry, causing mortality, respiratory symptoms, growth suppression and movement disorders. It is known for its ability to acquire resistance against various classes of antibiotic substances. In this study a protocol for antimicrobial susceptibility testing of *O. rhinotracheale* with microdilution for the commercially available MICRONAUT-S system was developed. Its results for 66 *O. rhinotracheale* strains were evaluated, proving that the protocol enabled sufficient growth and reliable measurement of MIC values for all, except one strain. Based on the developed protocol *O. rhinotracheale* strains isolated from Austrian turkey farms between 2002 and 2021 were investigated and classified based upon their phenotypic properties (colony size, heterogeneity, hemolysis and colour) and were tested for their antibiotic susceptibility. It was shown, that *O. rhinotracheale* isolates differed noticeably in their phenotypic appearance with colony sizes ranging from ≤ 0.3 mm to ≥ 2.0 mm and colour of colonies varying from grey to transparent and occasionally reddish. About 40 % of the isolates developed no hemolysis, while 45 % showed an incomplete hemolysis and over 15% of strains were able to cause β-hemolysis. The overall susceptibility to antimicrobial substances barely changed between 2002 and 2021, with resistance to the relevant antibiotics in poultry medicine being rather low. 1.5 % of strains were resistant to amoxicillin, 9.2% were resistant to enrofloxacin, 1.5% showed resistance against tetracycline and 12.3 % were not susceptible to tylosin. No resistance was found against sulfamethoxazole and the combination of trimethoprim and sulfamethoxazole. On the other hand, trimethoprim resistance was frequent with only 23.1% of isolates being susceptible and all strains were resistant to the tested aminoglycosides and to colistin. It was also shown, that *O. rhinotracheale* can acquire and lose resistance easily and quickly. Strains isolated from the same farm from successive outbreaks differed noticeably in their resistance profile with a slight tendency to gain resistance.

5.2 Zusammenfassung

O, rhinotracheale ist ein wichtiger Krankheitserreger beim Geflügel, welcher erhöhte Mortalität, respiratorische Symptome, vermindertes Wachstum und Bewegungsstörungen verursachen kann. Beschrieben ist außerdem seine Fähigkeit Resistenzen gegen verschiedene Antibiotikaklassen zu entwickeln. In der vorliegenden Studie wurde ein Protokoll für Resistenztestungen von *O. rhinotracheale* mittels Mikrodilution mithilfe des kommerziell erhältlichen MICRONAUT-S Systems entwickelt. Dessen Resultate für 66 O. rhinotracheale Isolate wurden evaluiert, wodurch gezeigt werden konnte, dass dieses Protokoll ausreichend Bakterienwachstum und eine verlässliche Messung von MHK Werten für alle, mit Außnahme eines Isolates ermöglichte. Außerdem wurden alle *O. rhinotracheale* Isolate, die zwischen 2002 und 2021 von österreichischen Betrieben isoliert wurden, bezüglich ihrer phenotypischen Eigenschaften untersucht (Koloniegröße, Heterogenität, Hämolyse und Farbe). Des Weiteren wurden Antibiotikaresistenztestungen durchgeführt. Es konnte gezeigt werden, dass sich O. rhinotracheale Isolate in ihrem Erscheinungsbild deutlich unterscheiden. Die Größe der Kolonien reicht von < 0,3 mm bis > 2,0 mm und die Farbe der Kolonien schwankt zwischen grau, transparent und manchmal rötlich. Ungefähr 40 % der Isolate zeigten keine Hämolyse, während knapp 45 % eine α-Hämolyse aufwiesen. Bei etwas über 15 % der Isolate wurde eine β-Hämolyse festgestellt. Die Wirksamkeit von Antibiotika veränderte sich von 2002 bis 2021 kaum und die Resistenzen gegen die relevanten Antibiotika in der Geflügelmedizin waren insgesamt auf einem niedrigen Niveau. 1,5 % der Isolate waren resistant gegen Amoxicilin, 9,2 % resistant gegen Enrofloxacin, 1,5 % zeigten Resistenzen gegen Tetrazyklin und 12,3 % gegen Tylosin. Keine Resistenzen wurden gefunden gegen Sulfamethoxazol und die Kombination von Trimethoprim und Sulfamethoxazol. Andererseits waren Resistenzen gegen Trimethoprim häufig mit 76,9 % und alle Isolate waren resistant gegen die getesteten Aminoglykoside und Colistin. Es konnte auch gezeigt werden, dass *O. rhinotracheale* sein Resistenzprofil verändern kann. Stämme, die bei wiederholten Ausbrüchen von dem selben Betrieb isoliert wurden, zeigten deutliche Unterschiede in ihrem Resistenzmuster und eine leichte Tendenz zu einer größeren Zahl an Resistenzen war zu erkennen.

6 References

Ak, S.; Turan, N. (2001): Antimicrobial susceptibility of Ornithobacterium rhinotracheale isolated from broiler chickens in Turkey. In *Vet. Arhiv* 71, pp. 121–127.

Alispahic, Merima; Christensen, Henrik; Bisgaard, Magne; Hess, Michael; Hess, Claudia (2014): MALDI-TOF mass spectrometry confirms difficulties in separating species of the Avibacterium genus. In *Avian pathology : journal of the W.V.P.A* 43 (3), pp. 258–263. DOI: 10.1080/03079457.2014.916038.

Alispahic, Merima; Christensen, Henrik; Hess, Claudia; Razzazi-Fazeli, Ebrahim; Bisgaard, Magne; Hess, Michael (2011): Identification of Gallibacterium species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry evaluated by multilocus sequence analysis. In *International journal of medical microbiology : IJMM* 301 (6), pp. 513–522. DOI: 10.1016/j.ijmm.2011.03.001.

Alispahic, Merima; Endler, Lukas; Hess, Michael; Hess, Claudia (2021): Ornithobacterium rhinotracheale: MALDI-TOF MS and Whole Genome Sequencing Confirm That Serotypes K, L and M Deviate from Well-Known Reference Strains and Numerous Field Isolates. In *Microorganisms* 9 (5). DOI: 10.3390/microorganisms9051006.

Baquer, Florian; Ali Sawan, Asma; Auzou, Michel; Grillon, Antoine; Jaulhac, Benoît; Join-Lambert, Olivier; Boyer, Pierre H. (2021): Broth Microdilution and Gradient Diffusion Strips vs. Reference Agar Dilution Method: First Evaluation for Clostridiales Species Antimicrobial Susceptibility Testing. In *Antibiotics (Basel, Switzerland)* 10 (8). DOI: 10.3390/antibiotics10080975.

Barbosa, Eunice Ventura; Cardoso, Clarissa Varajão; Silva, Rita de Cássia Figueira; Cerqueira, Aloysio de Mello Figueiredo; Liberal, Maíra Halfen Teixeira; Castro, Helena Carla (2019): Ornithobacterium rhinotracheale: An Update Review about An Emerging Poultry Pathogen. In *Veterinary sciences* 7 (1). DOI: 10.3390/vetsci7010003.

Brian J. Werth (2020): Trimethoprim and Sulfamethoxazole. MSD Manual. MSD. Available online at https://www.msdmanuals.com/professional/infectious-diseases/bacteria-and-antibacterial-drugs/trimethoprim-and-sulfamethoxazole, updated on May 2020, checked on 11/30/2021.

Brogden, R. N.; Carmine, A. A.; Heel, R. C.; Speight, T. M.; Avery, G. S. (1982): Trimethoprim: a review of its antibacterial activity, pharmacokinetics and therapeutic use in urinary tract infections. In *Drugs* 23 (6), pp. 405–430. DOI: 10.2165/00003495-198223060-00001.

Bundesamt für Sicherheit im Gesundheitswesen (2021): Arzneispezialitätenregister. Edited by Bundesamt für Sicherheit im Gesundheitswesen. Bundesamt für Sicherheit im Gesundheitswesen. Available online at https://aspregister.basg.gv.at/aspregister/faces/aspregister.jspx?_adf.ctrl-state=tl0ao77c7_4, updated on 12/16/2021, checked on 12/16/2021.

Churria, Carlos D. Gornatti; Loukopoulo, Panayiotis; Vigo, German B.; Sansalone, Pablo; Machuca, Mariana A.; Nievas, Victorio et al. (2016): In vitro Antibiotic Susceptibility Patterns of Ornithobacterium rhinotracheale from Commercial Chickens in Argentina#. In *International J. of Poultry Science* 15 (8), pp. 293–296. DOI: 10.3923/ijps.2016.293.296.

CLSI (2008): Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. In *Clinical an Laboratory Standards Institute* 4th ed. CLSI supplement VET08.

Cordovana, Miriam; Ambretti, Simone (2020): Antibiotic susceptibility testing of anaerobic bacteria by broth microdilution method using the MICRONAUT-S Anaerobes MIC plates. In *Anaerobe* (63). DOI: 10.1016/j.anaerobe.2020.102217.

European Commission (2009): Commission Regulation (EU) No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Source: http://extwprlegs1.fao.org/docs/pdf/eur92214.pdf.

Hafez, Hafez M.; Friedrich, S. (1998): Isolation of Ornithobacterium rhinotracheale from meat turkey in Austria. In *Tierärztliche Umschau* 53 (8), pp. 500–504.

Hafez Mohamed Hafez (2002): Diagnosis of Ornithobacterium Rhinotracheale. In *International J. of Poultry Science* 1 (5), pp. 114–118. DOI: 10.3923/ijps.2002.114.118.

Malik, Yashpal S.; Olsen, Karen; Kumar, Kuldeep; Goyal, Sagar M. (2003): In vitro antibiotic resistance profiles of Ornithobacterium rhinotracheale strains from Minnesota turkeys during 1996-2002. In *Avian diseases* 47 (3), pp. 588–593. DOI: 10.1637/6086.

Melter, O.; Radojevič, B. (2010): Small colony variants of Staphylococcus aureus--review. In *Folia microbiologica* 55 (6), pp. 548–558. DOI: 10.1007/s12223-010-0089-3.

MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH (Ed.): MICRONAUT-S product description. Microplates for automated or manual suscepitibility testing of bacteria. Available

online at https://www.merlin-diagnostika.de/fileadmin/mediapool/downloads/Produkte/Resistenzbestimmung/F_MCN-S_30032012_e.pdf, checked on 6/5/2021.

MERLIN Gesellschaft für mikrobiologische Diagnostika GmbH (Ed.): Micronaut-S Produktbeschreibung. Mikrotitrationsplatten für die automatisierte oder manuelle Empfindlichkeitsprüfung von Bakterien. Available online at https://www.merlin-diagnostika.de/fileadmin/mediapool/downloads/Produkte/Resistenzbestimmung/F_MCN-S_30032012.pdf, checked on 6/5/2021.

Mohd-Zain, Z.; Lin Jee, T.; Jusoff, K. (2008): Phenotypic Characteristics, Antibiotic Susceptibility and Pathogenicity of Ornithobacterium rhinotracheale. In *WSEAS Transact. Biol. Biomed* 7, pp. 133–142.

Murthy, T.R.G.K.; Dorairajan, N.; Balasubramanium, G. A.; Dinakaran, A. M.; Saravanabava, K. (2008): In vitro antibiotic sensitivity of Ornithobacterium rhinotracheale strains isolated from laying hens in India. In *Vet. Arhiv* 78, pp. 49–56.

Peña-Vargas, Edgar Rafael; Vega-Sánchez, Vicente; Morales-Erasto, Vladimir; Trujillo-Ruíz, Héctor Hugo; Talavera-Rojas, Martín; Soriano-Vargas, Edgardo (2016): Serotyping, Genotyping, and Antimicrobial Susceptibility of Ornithobacterium rhinotracheale Isolates from Mexico. In *Avian diseases* 60 (3), pp. 669–672. DOI: 10.1637/11333-112515-ResNote.1.

Pfennigwerth, N.; Kaminski, A.; Korte-Berwanger, M.; Pfeifer, Y.; Simon, M.; Werner, G. et al. (2019): Evaluation of six commercial products for colistin susceptibility testing in Enterobacterales. In *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 25 (11), pp. 1385–1389. DOI: 10.1016/j.cmi.2019.03.017.

Proctor, R. A.; Kahl, B.; Eiff, C. von; Vaudaux, P. E.; Lew, D. P.; Peters, G. (1998): Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. In *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 27 Suppl 1, S68-74. DOI: 10.1086/514906.

Proctor, Richard A.; Eiff, Christof von; Kahl, Barbara C.; Becker, Karsten; McNamara, Peter; Herrmann, Mathias; Peters, Georg (2006): Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. In *Nature reviews. Microbiology* 4 (4), pp. 295–305. DOI: 10.1038/nrmicro1384.

Siegmann, Otfried (Ed.) (2011): Kompendium der Geflügelkrankheiten. Unter Mitarbeit führender Spezialisten aus Lehre, Praxis und Forschung. 7., überarbeitete Auflage. Hannover: Schlütersche. Available online at https://content-select.com/index.php?id=bib&ean=9783842683334.

Sugino, A.; Peebles, C. L.; Kreuzer, K. N.; Cozzarelli, N. R. (1977): Mechanism of action of nalidixic acid: purification of Escherichia coli nalA gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. In *Proceedings of the National Academy of Sciences of the United States of America* 74 (11), pp. 4767–4771. DOI: 10.1073/pnas.74.11.4767.

Swayne, David E. (Ed.) (2020): Diseases of Poultry. Fourteenth edition. Hoboken, NJ: Wiley-Blackwell.

Szabó, Réka; Wehmann, Enikő; Magyar, Tibor (2015): Antimicrobial susceptibility of Bordetella Avium and Ornithobacterium Rhinotracheale strains from wild and domesticated birds in Hungary. In *Acta veterinaria Hungarica* 63 (4), pp. 413–424. DOI: 10.1556/004.2015.039.

Tabatabai, Louis B.; Zimmerli, Mandy K.; Zehr, Emilie S.; Briggs, R. E.; Tatum, Fred M. (2010): Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. In *Avian diseases* 54 (3), pp. 994–1001. DOI: 10.1637/9070-091409-Reg.1.

Tsai, Hsiang-Jung; Huang, Chen-Wei (2006): Phenotypic and molecular characterization of isolates of Ornithobacterium rhinotracheale from chickens and pigeons in Taiwan. In *Avian diseases* 50 (4), pp. 502–507. DOI: 10.1637/7527-031906R.1.

van Empel, P.; van den Bosch, H.; Loeffen, P.; Storm, P. (1997): Identification and serotyping of Ornithobacterium rhinotracheale. In *J Clin Microbiol* 35 (2), pp. 418–421. DOI: 10.1128/jcm.35.2.418-421.1997.

van Empel, P. C.; Hafez, H. M. (1999): Ornithobacterium rhinotracheale: A review. In *Avian pathology : journal of the W.V.P.A* 28 (3), pp. 217–227. DOI: 10.1080/03079459994704.

van Veen, L.; Hartman, E.; Fabri, T. (2001): In vitro antibiotic sensitivity of strains of Ornithobacterium rhinotracheale isolated in The Netherlands between 1996 and 1999 149 (20), pp. 611–613. DOI: 10.1136/vr.149.20.611.

Vandamme, P.; Segers, P.; Vancanneyt, M.; van Hove, K.; Mutters, R.; Hommez, J. et al. (1994): Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. In *International journal of systematic bacteriology* 44 (1), pp. 24–37. DOI: 10.1099/00207713-44-1-24.

Walters, J.; Evans, R.; LeRoith, T.; Sriranganathan, N.; McElroy, A.; Pierson, F. W. (2014): Experimental comparison of hemolytic and nonhemolytic Ornithobacterium rhinotracheale field isolates in vivo. In *Avian diseases* 58 (1), pp. 78–82. DOI: 10.1637/10637-081313-Reg.1.

Zahra, Mohammad; Ferreri, Miro; Alkasir, Rashad; Yin, Jinhua; Han, Bo; Su, Jingliang (2013): Isolation and characterization of small-colony variants of Ornithobacterium rhinotracheale. In *J Clin Microbiol* 51 (10), pp. 3228–3236. DOI: 10.1128/JCM.01337-13.

7 Illustration directory

8 Attachment

Attachment 1 *Results Test cycle 4 strain 02/103*

	02/103 McF 0,5 24h	02/103 McF 0,5 48h	02/103 McF 2 24h	02/103 McF 2 48h
Amoxicillin + CS	S	S	S	S
Ampicillin	S	S	S	S
Amoxicillin	S	S	S	S
Ceftazidim	R	R	R	R
Cefazolin	R	R	R	R
Chloramphenicol	S	S	S	S
Colistin	R	R	R	R
Cefoxitin	S	S	S	S
Cefotaxim	I	R	R	R
Enrofloxacin	I	I	I	I
Gemtamicin	R	R	R	R
Imipenem	S	S	S	S
Nalidixinsäure	I	R	R	R
Neomycin	R	R	R	R
Oxacillin	R	R	R	R
Sulfamethoxazol	S	S	S	S
Streptomycin	R	R	R	R
Trimethoprim / Sulfamethoxazol	S	S	S	S
Trimethoprim / Sulfamethoxazol Harninf.	S	S	S	S
Trimethoprim / Sulfamethoxazol system. Inf.	R	R	R	R
Tetracyclin	I	I	I	I
Tylosin	S	S	S	R
Trimethoprim	S	R	S	R

Attachment 2 *Results Test cycle 4 strain 02/301*

	02/301 McF 0,5 24h	02/301 McF 0,5 48h	02/301 McF 2 24h	02/301 McF 2 48h
Amoxicillin + CS	S	S	S	S
Ampicillin	S	S	S	S
Amoxicillin	S	S	S	S
Ceftazidim	S	S	S	S
Cefazolin	S	S	S	S
Chloramphenicol	S	S	S	S
Colistin	R	R	R	R
Cefoxitin	S	S	S	S
Cefotaxim	S	S	S	S
Enrofloxacin	I	I	I	I
Gemtamicin	R	R	R	R
Imipenem	S	S	S	S
Nalidixinsäure	R	R	R	R
Neomycin	R	R	R	R
Oxacillin	R	R	R	R
Sulfamethoxazol	S	S	S	S
Streptomycin	R	R	R	R
Trimethoprim / Sulfamethoxazol	S	S	S	S
Trimethoprim / Sulfamethoxazol Harninf.	S	S	S	S
Trimethoprim / Sulfamethoxazol system. Inf.	R	R	R	R
Tetracyclin	S	S	S	S
Tylosin	S	S	S	S
Trimethoprim	R	R	R	R

Attachment 3 *Results Test cycle 4 strain 02/658*

	02/658 McF 0,5 24h	02/658 McF 0,5 48h	02/658 McF 2 24h	02/658 McF 2 48h
Amoxicillin + CS	S	S	S	S
Ampicillin	S	S	S	S
Amoxicillin	S	S	S	S
Ceftazidim	S	S	S	S
Cefazolin	S	S	S	S
Chloramphenicol	S	S	S	S
Colistin	R	R	R	R
Cefoxitin	S	S	S	S
Cefotaxim	S	S	S	S
Enrofloxacin	I	I	I	I
Gemtamicin	R	R	R	R
Imipenem	S	S	S	S
Nalidixinsäure	I	R	I	R
Neomycin	R	R	R	R
Oxacillin	S	S	S	S
Sulfamethoxazol	S	S	S	S
Streptomycin	R	R	R	R
Trimethoprim / Sulfamethoxazol	S	S	S	S
Trimethoprim / Sulfamethoxazol Harninf.	S	S	S	S
Trimethoprim / Sulfamethoxazol system. Inf.	R	R	R	R
Tetracyclin	S	S	S	S
Tylosin	S	S	S	S
Trimethoprim	S	R	R	R