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Antimicrobial Dilution Susceptibility Testing of *Erysipelothrix rhusiopathiae* According to CLSI Document VET06 Reveals High Resistance against Penicillin G, Erythromycin and Enrofloxacin

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Abstract: Erysipelas is a re-emerging disease in different poultry species. Antibiotic treatment is crucial to combat outbreaks in poultry flocks, but only very limited data on susceptibility are available. Recently, the Clinical and Laboratory Standard Institute established standardized guidelines and minimal inhibitory concentration breakpoints for *E. rhusiopathiae* when using the broth microdilution method. In the present investigation, these guidelines were applied to evaluate the antimicrobial susceptibility of 30 *E. rhusiopathiae* isolates derived from field outbreaks in poultry flocks towards penicillins, macrolides, lincosamides and fluoroquinolones. All isolates were identified by MALDI-TOF MS. The majority of isolates belonged to two serovars, 1b and 5. More than 40% of the isolates proved resistant to penicillin G, with values ranging from 0.25 to 8 μg/mL. Furthermore, the majority of isolates were found resistant to erythromycin (76.7%; MIC 2–4 μg/mL) and enrofloxacin (60.0%; MIC ≥ 2 μg/mL), altogether limiting treatment options. In contrast, most of the isolates proved susceptible to ampicillin and ceftiofur with MICs ≤ 0.25 μg/mL and ≤ 2 μg/mL, respectively. A great variety of antimicrobial resistance patterns was found, and multidrug resistance was detected in one-third of the isolates. The presented data are helpful to raise awareness for the antimicrobial resistance of a zoonotic pathogen in context of the One Health concept.

Keywords: antibiotics; susceptibility; erysipelas; One Health

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1. Introduction

Erysipelas is an acute septicemic disease affecting a variety of avian species [1]. The causative agent is *Erysipelothrix* (*E.*) *rhusiopathiae*, a Gram-positive, rod-shaped, non-motile bacterium. Outbreaks of erysipelas in poultry flocks are mainly characterized by sudden death of birds often without accompanying clinical signs. This coincides with high morbidity and mortality, leading to severe economic losses [2]. Gross lesions are characterized by hepatosplenomegaly often with white pinhead-sized necrotic nodules. Furthermore, catarrhal to hemorrhagic-mucoid enteritis is reported [1]. Diagnosis is based on the isolation of the agent from affected organs on blood agar, and the use of a microaerophilic milieu is recommended to enhance growth. Colonies of *E. rhusiopathiae* are very small (0.3–1.5 mm) and appear often with an α-hemolysis [1]. Until today, classical phenotypic features, such as morphology of in vitro culture, Gram-staining and biochemical tests, are used for identification. However, in recent years, protein profiling of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was successfully implemented for identification [3,4]. In total, 16 serovars of *E. rhusiopathiae* are defined [5], and in poultry dominant serovars are 1a, 1b, 4, 5, 6, 15 and N [2]. In recent years, erysipelas became a re-emerging infectious disease in commercial poultry flocks based on the trend to keep birds outside leading to exposure with carriers, such as wild fowl, rodents

and insects, but also environmental sources [6–10]. For example, the accumulation of the agent in the environment, the contaminated manure and dust were shown to represent sources of transmission [10]. As only a limited number of commercial vaccines are available, autogenous vaccines are widely used. Nonetheless, disease outbreaks in vaccinated flocks have been reported [10]. Therefore, antibiotic treatment of affected flocks is crucial to control and eliminate the disease, and mainly penicillins are used. Currently, less actual information is available regarding the antibiotic susceptibility of *E. rhusiopathiae* isolates, which may not only lead to treatment failures in poultry flocks, but can also be a source of antibiotic-resistant isolates that may represent a risk for human health considering that the bacteria are a zoonotic agent.

Therefore, the aim of this study was to provide more data on the antibiotic susceptibility patterns of *E. rhusiopathiae*. For this purpose, 30 isolates derived from outbreaks in Austrian poultry flocks over a time period from 2003–2021 were investigated to determine the antimicrobial resistance profile. By applying a standardized protocol providing guidance to veterinary diagnostics regarding susceptibility testing of *E. rhusiopathiae* [11], the study enhances comparability of data recruited in future. This shall not only contribute to targeted treatments of affected poultry flocks but also assist to monitor trends in antibiotic resistance development.

2. Materials and Methods

2.1. E. rhusiopathiae Isolates

From 2003 to 2021, a total of 30 *E. rhusiopathiae* isolates were obtained from field outbreaks of erysipelas in poultry flocks and stored at −80 °C in 2 mL tubes with 40% glycerol/10 mL Brain Heart Infusion Broth (Oxoid©, ThermoFisher Scientific, Wien, Austria). Necropsies and bacteriological investigations were performed at the Clinic for Poultry and Fish Medicine, Veterinary University Vienna (Vienna, Austria) in cooperation with the veterinarians in charge of the respective flocks having agreements for the application of veterinary procedures with the farmers. The outbreaks were unrelated to each other, each isolate was derived from a single farm. In total, 16 outbreaks occurred in layer hen flocks, 12 in turkey flocks and 1 in a geese flock. From isolate no. 11, information regarding the source, clinical symptoms and pathological lesions was not available (Table 1). The outbreaks were characterized by sudden death of birds, and gross pathology revealed in all birds' hepatosplenomegaly with pinpoint-sized white necrotic nodules. Furthermore, in the majority of outbreaks (24/29), mucoid-hemorrhagic enteritis was found. For the actual investigations, isolates were thawed and cultivated on blood agar (Columbia agar containing 5% sheep blood, BioMeriéux, Vienna, Austria) at 37 °C for 48 h under microaerophilic conditions (Genbox microaer, BioMèrieux, Vienna, Austria).

Table 1. *E. rhusiopathiae* isolates according their origin, serovar, MALDI-TOF MS identification score and antimicrobial susceptibility against the six tested substances.

No.	Isolate	Origin	Serovar	MALDI Score	Antimicrobial Substance					
					PEN [b)]	AMP [c)]	CEF [d)]	ERY [e)]	CLI [f)]	ENRO [g)]
1	PA03/01999 [a)]	Laying hens	5	2.23	S	S	S	S	S	S
2	PA04/02665	Laying hens	1b	2.01	S	S	S	R	R	R
3	PA04/02706	Laying hens	1b	2.26	S	S	S	S	S	S
4	PA04/03029	Laying hens	5	2.17	S	S	S	S	R	R
5	PA04/03411	Laying hens	1b	2.23	S	S	S	R	R	I
6	PA04/03467	Laying hens	5	2.11	S	S	S	R	R	R
7	PA04/03961	Laying hens	2	2.03	R	S	S	R	R	R
8	PA04/04254	Laying hens	5	2.32	R	S	S	R	R	R
9	PA06/02766	Laying hens	1b	2.05	S	S	S	R	R	R
10	PA06/02769	Laying hens	1b	2.22	S	S	S	R	R	R
11	PA06/06830	No information	5	2.23	S	S	S	R	R	R
12	PA11/02519	Turkeys	5	2.18	S	S	S	R	R	R
13	PA12/01398	Turkeys	5	2.09	S	S	S	R	R	R
14	PA12/13711	Turkeys	5	2.21	S	S	S	R	R	I
15	PA13/11559	Turkey	5	2.13	R	R	R	S	R	I
16	PA13/19126	Turkeys	5	2.10	R	R	R	R	R	R
17	PA13/20681	Laying hens	1b	2.30	S	S	S	R	S	S
18	PA14/03494	Laying hens	1b	2.17	R	S	S	S	S	I
19	PA16/05917	Laying hens	1b	2.02	S	S	S	I	S	S
20	PA16/20064	Turkeys	1b	2.17	R	R	R	R	R	R
21	PA16/23313	Turkeys	5	2.22	S	S	S	R	S	R
22	PA17/06068	Laying hens	1b	2.15	S	S	S	R	S	R
23	PA17/13772	Turkeys	1b	2.09	R	S	S	R	I	I

Table 1. *Cont.*

No.	Isolate	Origin	Serovar	MALDI Score	Antimicrobial Substance					
					PEN [b)]	AMP [c)]	CEF [d)]	ERY [e)]	CLI [f)]	ENRO [g)]
24	PA17/22701	Laying hens	1b	2.14	R	S	S	R	S	R
25	PA17/22709	Turkeys	1b	2.28	R	R	S	R	S	R
26	PA17/25575	Turkeys	5	2.28	R	R	S	R	S	R
27	PA20/24032	Geese	5	2.08	S	S	S	R	S	I
28	PA21/01504	Turkeys	5	2.19	R	R	S	R	S	R
29	PA21/05003	Laying hens	5	2.03	R	S	S	R	S	R
30	PA21/18180	Turkeys	5	2.21	S	S	S	I	S	S

[a)] PA year of isolation/sample number; [b)] penicillin G; [c)] ampicillin; [d)] ceftiofur; [e)] erythromycin; [f)] clindamycin; [g)] enrofloxacin.

2.2. Identification and Serotyping of Isolates

For identification all isolates were subjected to MALDI-TOF MS using MALDI Biotyper sirius and the supplied software MBT Compass 4.3 (Bruker Daltonics, Bremen, Germany). Samples were prepared by the direct transfer method according to the protocol of the manufacturer. Briefly, one to two single colonies were transferred onto a steel target plate by using disposable 1 µL loops (VWR, Vienna, Austria). 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. For species identification, the MALDI Biotyper sets 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. A MALDI score between 1.7 and 2.0 correlates with 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.

Serotyping of isolates was performed by multiplex PCR according to the protocol from Shimoji et al. [12]. DNA extraction of isolates was performed by the DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Multiplex PCR was performed in a 25 µL reaction mixture using the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) using a 25 ng DNA template. The thermal profile was as follows: initial denaturation for 15 min at 95 °C, followed by 30 amplification cycles of a denaturing step at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min; and one cycle final extension at 72 °C for 10 min. The PCR products were visualized by agarose gel electrophoresis.

2.3. Antimicrobial Susceptibility Test

The broth microdilution method was applied according to CLSI supplement VET06 [11] to test the antimicrobial sensitivity of the *E. rhusiopathiae* isolates using a commercially available plate MICRONAUT-S Lifestock/Equines GP (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany). The antimicrobial substances, their concentrations and the used cut-offs for intermediate and resistant strains are given in Table 2.

Table 2. Antimicrobial substances and concentrations used for AMR testing including the respective minimal inhibitory concentration (µg/mL) for intermediate (light grey) and resistant strains (bold, dark grey) and the number of sensitive, intermediate and resistant strains.

Class	Antimicrobial Substance	Concentration (µg/mL)							No. of Strains S/I/R [a]
Penicillins	Penicillin G	0.125	**0.25**	0.5	1	2	4	8	17/n.d. [b]/13
	Ampicillin	0.125	0.25	**0.5**	1	2	4	8	24/n.d./4
Cephalosporins (3rd generation)	Ceftiofur	0.125	0.25	0.5	1	2	4		27/3/0
Macrolides	Erythromycin	0.125	0.25	0.5	**1**	2	4		5/2/23
Fluoroquinolones	Enrofloxacin	0.0625	0.125	0.25	0.5	1	**2**		6/6/18
Lincosamides	Clindamycin	0.125	0.25	0.5	**1**	2			27/1/2

[a] Sensitive/Intermediate/Resistant; [b] not defined.

The preparation of the bacterial test suspensions was done according to the CLSI instructions. Briefly, direct colony suspension was performed in Mueller Hinton II broth supplemented with horse blood (Liofilchem, Roseto degli Abruzzi, Italy). Each well of the MICRONAUT-S Lifestock/Equines GP plate was inoculated with 100 µL of the bacterial suspension. Afterwards, plates were incubated microaerobically for 24 h at 37 °C. The evaluation of the results was done visually by eye according to the breakpoints for broth microdilution susceptibility testing of CLSI supplement VET06 [12].

The minimum inhibitory concentration (MIC) was the lowest concentration of an antimicrobial substance, which completely prevented growth. Multidrug resistance was defined as resistance to at least one substance in three or more antimicrobial classes.

3. Results

All isolates were identified as *E. rhusiopathiae* by MALDI-TOF MS with identification levels above score value 2.0. Serotypes 1b and 5 were most prevalent: serotype 1b comprised 13 isolates, while serotype 5 comprised 16 isolates. One isolate could be classified as serotype 2. Heterogeneous antimicrobial susceptibility patterns were found when isolates were tested against penicillin G, ampicillin, ceftiofur, erythromycin, clindamycin and enrofloxacin (Table 1).

Within the group of penicillins, a clear difference in resistance was observed when comparing the results from penicillin G to ampicillin. In total, 42.3% (n = 13) of the strains proved resistant to penicillin G with MIC values ranging from 0.25–8 μg/mL. In contrast, only 13.3% (n = 4) showed resistance to ampicillin (MICs $\geq$ 8 μg/mL). Similarly, the majority of isolates (90%, n = 27) were susceptible to ceftiofur. Most isolates proved resistant to erythromycin (76.7%, n = 23) and enrofloxacin (60.0%, n = 18) with MIC values ranging from 2–4 μg/mL and $\geq$2 μg/mL, respectively. Full susceptibility to clindamycin was found in 90% (n = 27) of the isolates (Table 2).

The 30 *E. rhusiopathiae* isolates represent 11 antimicrobial resistance patterns. Most of the isolates (43.3%) grouped into patterns 4 and 7 with the combinations penicillin G, erythromycin, enrofloxacin (PEN-ERY-ENRO) and erythromycin, enrofloxacin (ERY-ENRO), respectively (Table 3). Ten isolates proved as multidrug-resistant all of them harboring resistance to penicillins, macrolides and fluoroquinolones. Out of these, two isolates were non-susceptible to all antimicrobials tested.

Table 3. Antimicrobial resistance patterns of isolates organized by antimicrobial substances.

Resistance Pattern	Antimicrobial Substances	No. of Isolates
1	**PEN-AMP-CEF-ERY-CLI-ENRO**	2
2	**PEN-AMP-ERY-ENRO**	3
3	**PEN-AMP-CEF-***ENRO*	1
4	**PEN-ERY-ENRO**	5
5	**PEN-ERY**	1
6	**PEN-***ENRO*	1
7	**ERY-ENRO**	7
8	**ERY-***ENRO*	3
9	**ERY**	1
10	**ENRO**	2
11	*ERY*	2

ERY: erythromycin, ENRO: enrofloxacin, PEN: penicillin G, AMP: ampicillin, CEF: ceftifour, CLI: clindamycin; bold: resistant, italics: intermediate.

4. Discussion

Today, erysipelas is considered as re-emerging disease in different poultry species and antibiotic treatment is crucial to effectively combat the disease, with penicillin as the drug of choice [1,2]. Thus far, only limited data on antibiotic susceptibility in *E. rhusiopathiae* are available. Therefore, there is a clear need to monitor antibiotic resistance of isolates, which will be helpful and necessary to optimize a targeted therapy. With the document VET06 [12], breakpoints for fastidious bacteria such as *E. rhusiopathiae* are provided for veterinary use. Based on this, we evaluated six listed antimicrobial agents belonging to four classes against thirty *E. rhusiopathiae* isolates by broth microdilution susceptibility testing.

The isolates were successfully identified to species level by MALDI-TOF MS, which is in agreement with previous studies [3,10,13]. The majority of isolates belonged to serotypes 1 and 5, which is congruent to earlier investigations [8,14–17]. The drug of choice for treatment is still penicillin, but varying success has been reported in affected poultry

flocks [1,14]. Interestingly, so far, isolates with low MICs $\leq$ 0.12 µg/mL against penicillin G were reported [8,18]. This is in clear contrast to the present findings with over 40% resistant isolates with clearly higher MIC ranges. In this context, beside penicillin binding proteins (PBPs), ß-lactamases are also found of high importance in Gram-positive bacteria for underlying resistance mechanisms [19]. Interestingly, most of the isolates were susceptible to ampicillin and ceftiofur, although a similar resistance rate would be expected within the group of ß-lactams. Such unusual resistance phenotypes have been reported from *Enterococcus faecalis* isolates, which are most likely based on point mutations in genes encoding PBPs, a feature which could be present in *E. rhusiopathiae* isolates too and should be explored in future studies [20–23]. Macrolides are recommended as alternative medication in case of treatment failures with penicillins. Previous studies revealed increasing numbers of macrolide-resistant isolates from pigs, which is plasmid mediated [24–27]. In the present study, most isolates proved resistant to erythromycin with MICs clearly above the breakpoints supplied by the CLSI supplement VET06 [12], which is in agreement with resistance data from poultry reported by Eriksson et al. [8]. Mutations in quinolone resistance-determining regions of *gyrA* and *parC* are known as important mechanisms to reduce the susceptibility in *E. rhusiopathiae* and might be an explanation for the current finding for enrofloxacin with MICs $\geq$ 2 µg/mL [27]. This is in clear contrast to resistance data reported over nearly the last two decades where low resistance rates with MICs ranging from $\leq$ 0.12 µg/mL to 0.25 µg/mL were reported [8,28,29]. Whereas increasing resistance to clindamycin is reported from pig isolates, the majority of the present poultry isolates proved susceptible [27]. The antimicrobial resistance patterns of *E. rhusiopathiae* found here demonstrated a heterogeneous picture with 11 different resistance phenotypes independent of origin, serovar or year of isolation, which is in contrast to earlier reports on swine isolates [28]. However, a multidrug-resistant *E. rhusiopathiae* strain carrying several acquired antimicrobial resistance genes derived from a pig was recently identified [26], indicating a trend change in pig isolates too. In the presented study, one-third of the isolates were characterized as multidrug resistant, a finding which demands attention in future investigations.

Taken together, we provide antimicrobial susceptibility data on *E. rhusiopathiae* isolates from poultry based on the standardized guidelines by the CLSI supplement VET06 [12]. This shall not only assist targeted treatments of affected poultry flocks, but also contribute to monitor the trend in the development of antibiotic resistance in regard to the One Health concept.

5. Conclusions

An increase in resistance could be shown towards penicillin G, erythromycin and enrofloxacin with consequences on treatment options. Moreover, a large assortment of heterogeneous antimicrobial resistance patterns was found within multidrug-resistant isolates. Therefore, a continuous monitoring of antibiotic resistance in *E. rhusiopathiae* isolates according to standard protocols is advisable to gain an overview of the current situation in the field.

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