Molecular typing of Riemerella anatipestifer serotype 14, an emerging pathogen for ducks

Zur molekularen Typisierung von Feldstämmen von Riemerella anatipestifer Serotyp 14, einem Krankheitserreger mit zunehmender Bedeutung für Enten

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Introduction

Riemerella anatipestifer is a Gram-negative, microaerophilic, non-motile, rod-shaped bacterium causing septicemia and infectious seborrhea in a wide variety of wild and domestic birds (Sandhu, 2003). R. anatipestifer could be isolated from asymptomatic ducks (Frommer et al., 1990; Ryll et al., 2001b). However, case reports and experimental studies prove the virulence of R. anatipestifer for ducks and its role as a primary pathogen (Floren and Kælta, 1988; Fulton and Rimler, 2010; Sæver et al., 2005; Yu et al., 2008). Vaccination programs based on autogenous vaccines are widely used to protect farmed ducks (Floren and Kælta, 1988). At present, no commercial vaccines against R. anatipestifer are available. High antigenic variability displayed by the bacterium is reflected by identification of 21 serotypes so far (Busgaard, 1982; Pathanasophon et al., 2002). The efficacy of autogenous vaccines largely depends on the composition of the antigens and optimum protection is achieved only against homologous challenge (Huang et al., 2002; Pathanasophon et al., 1996). Usually, the vaccines contain inactivated R. anatipestifer from different serotypes which had been isolated from the farms of destina-
tion. The cases and bacterial isolates included in this study are derived from clinical specimens and cultures related to farms belonging to integrators in four regions (northwest Germany, northeast Germany, south Germany and Hungary). As the data on the clinical background and vaccine history of the samples submitted to a contract diagnostic laboratory is usually incomplete, only the information on the geographical origin of the samples was considered as valid data in this study. However, it can be assumed that only material from clinically diseased ducks was submitted. To be able to formulate vaccines individually for the farms of destination, R. anatipestifer are continuously isolated from clinical cases. Whereas serotypes 1, 2, 3, 5 and 15 have been described as frequent findings in ducks (Pathanasophon et al., 1994; Sandhu and Leister, 1991), serotype 14 represents a major and emerging propor-
tion of the R. anatipestifer isolations investigated in this study. Serotype 14 has earlier been detected in goose by Köhler et al., 1995. This particular serotype was detected more frequently and represented a higher proportion of the total isolates in the recent years. This lead to the decision to perform further typing of the isolates belonging to serotype 14 to gain insight in the intra-serotype variability and possible role in the epizootiology in duck populations.

We used two approaches for subtyping: pulsed-field gel electrophoresis (PFGE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The molecular characterization of bacterial isolates by PFGE is a highly discriminating, well-established method which has been applied for typing European (Kiss et al., 2007) and Asian R. anatipestifer strains (Yu et al., 2008). By using a protocol developed by Sun et al., 2012, it was expected to generate a suitable number of genome fragments by restriction with the endonuclease Smal. In order to obtain additional data and to compare the typing results, mass spectra were generated from bacterial proteins by MALDI-TOF MS. The role and availability of MALDI-TOF MS in routine veterinary bacteriology has developed rapidly during the past years (Bizzini et al., 2010). Diagnostic laboratories can identify bacteria in a fast, cost-effective and reliable manner with commercial mass spectrometry systems (Seng et al., 2009) on species- or at least at genus-level. The method is highly efficient and superior to biochemical in the identification of Riemerella species (Rubenstroth et al., 2012). In order to confirm the accuracy of the previous identification results of the isolates used in this study, we subjected the 49 R. anatipestifer serotype 14 isolates and 2 isolates belonging to serotypes 12 and 17, respectively, to mass spectrometry. Main spectra were generated for all isolates by repeated measuring of replicates from the individual samples and compared to determine their relatedness. The aims of this study were to evaluate the diversity of R. anatipestifer belonging to the emerging serotype 14 and to explore if further typing of a serotype could be of practical use for the selection seed material for the production of autogenous vaccines.

Materials and Methods

Bacterial strains

The 51 bacterial isolates used in this study are listed in Table 2. They were identified as R. anatipestifer by biochemical testing using API 20NE (BioMérieux, Nürtingen; Germany) according to Hinz et al., 1998. Serotyping was performed with typing sera from Biovac (Biovac, Beaucouzé, France). Typing sera against serotypes A, 1, 8, 12 and 14 were applied consecutively to identify positive agglutination results. Isolates which did not react with any of these

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Genotyping by PFGE analysis

PFGE was performed using a CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) using a modification of a protocol described by Sun et al. (2012). *R. anatipestifer* were incubated on standard 1 nutrient agar (Merck) at 37°C and 5% CO₂ for 24 h. Agarose plugs were made according to the PulseNet standard protocol (Risor et al., 2006) with slight modifications. Colonies were suspended in cell suspension buffer (50 mM Tris, 50 mM EDTA, 1 mg ml⁻¹ proteinase K) and PFGE was performed. Five distinct band patterns were obtained and designated A-E with sub-pattern A1 being closely related to pattern A; and three subpatterns (C1, C2, C3) being closely related to pattern C (Table 2, Fig. 1). Four strains originating from Hungary, in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to cast plugs, which were incubated with cell lysis buffer (50 mM Tris, 50 mM EDTA, 1% lauryl sarcosine, 0.1 mg ml⁻¹ proteinase K) at 54°C for 1.5 hours. After washing three times with distilled water at 50°C for 10 min, plugs were washed four times in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 50°C for 10 min. Plugs of genomic DNA were digested with *Smal* (Fermentas, St. Leon-Rot) at 37°C for 3 h. One percent agarose gels were run with 0.5 x TBE running buffer (89 mM Tris-borate, 1 mM EDTA) at 14°C under conditions of 6.0 V/cm with an initial/final switch time of 2.2/54.2 seconds and an angle of 120° for 20 h. The resulting gel was stained with Ethidium bromide for 30 min, destained in distilled water for 5 min and photographed under UV-illumination.

A similarity matrix (Free Tree 0.9.1.50, 1999) and a dendrogram (Tree View 1.6.6., 2001) were constructed by the unweighted pair group method with the arithmetic average (UPGMA) using the Dice similarity coefficient and a maximum position tolerance of 1% for the banding migration distance. The PFGE patterns were interpreted based on the criteria described by Tenover et al., 1995.

Results

Forty nine *R. anatipestifer* serotype 14 isolates were digested with *Smal* and PFGE was performed. Five distinct band patterns were obtained and designated A-E with sub-pattern A1 being closely related to pattern A; and three subpatterns (C1, C2, C3) being closely related to pattern C (Table 2, Fig. 1). Four strains originating from Hungary,
### Table 2. Table of *R. anatipestifer* strains used in this study (nd: not done)

<table>
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<th>serotype</th>
<th>PFGE pattern</th>
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formed pattern A1 which contained no strain from any other region. Pattern C including the subpatterns C1, C2 and C3 contained more of the previous isolates from northwest Germany dating from 2008 and only few recent isolates. Pattern A represents the largest group with 32 isolates from northern Germany (the origin from two strains is unknown). Pattern A isolates were not isolated before 2010 and were not detected in strains from any of the other regions. Pattern B was only detected once. Pattern D is represented by a serotype 12 strain of turkey origin and pattern E by a serotype 17 strain. Both isolates were used as controls.

Figure 2 shows the genetic relatedness of the PFGE-subtypes. Patterns A, B and C including their respective subpatterns are related to each other and are considerably distant to patterns D and E representing the deviating serotypes 12 and 17, respectively.

For the construction of the dendrograms based on the results from MALDI-TOF MS, the data were processed with default software settings (Fig. 3, Fig. 4). Fig. 3 shows that the serotype 14 strains of R. anatipestifer are closely related and cluster separately from the R. anatipestifer reference strains databank entries. Representing an outlying bacterial species, several E. coli reference data sets are included. Figure 4 is a detailed view of all R. anatipestifer mass spectra generated from this study. Forty nine serotype 14 isolates have distance levels > 300 from the two deviating serotypes 12 and 17. They form eight closely related clusters. The distribution of the strains was different from the results obtained by PFGE. The strains belonging to the same PFGE-group segregated in a non-systematical manner into different MALDI-TOF-MS clusters.

**Discussion**

The isolation of R. anatipestifer from clinical specimens requires some experience and the supplementation of media with antibiotics is beneficial for primary isolation (SANDHU, 2003). The identification of suspicious bacterial colonies by conventional phenotypic methods used to be laborious and time-consuming. Improved methods have been developed (HINZ et al., 1998; HAN et al., 2011) and the availability of MALDI-TOF MS nowadays is a great advantage in time and cost for diagnostic laboratories. In our study, all 49 R. anatipestifer serotype 14 and 2 control strains were correctly identified which confirms the reliability of the method under the conditions of a routine laboratory.

The high antigenic diversity of R. anatipestifer is reflected by the increasing number of serotypes which have been established during the past decades (SANDHU and LEISTER, 1991). More serotypes will emerge as untypable isolates initially detected in the course of systematic investigations and in routine diagnostics (METZENER et al., 2008; SANDHU and LEISTER, 1991). In clinical outbreaks, it is common to detect more than one serotype (Yu et al., 2008). The use of autogenous vaccines is an appropriate tool for the prevention of Riemerellosis (LAYTON and SANDHU, 1984; HUANG et al., 2002). The emergence of formerly rare or unknown bacterial strains as described here might be triggered by vaccination programmes. Continuous diagnosis is required to ensure that the produced vaccines cover all relevant R. anatipestifer strains and provide homologous protection. Serotyping is a very important tool, but the method needs to be updated continuously and commercial typing sera are required for reproducible results (PATHANASOPHON et al., 2002). In our study, serotype 14 represented a major and emerging proportion of R. anatipestifer field isolates. PFGE-subtyping within this serotype resulted in 9 distinct band patterns. The interpretation of the resulting pattern allowed some conclusions with practical value for the selection of seed material for vaccine production: all four isolates from one geographical region distant from the other three were similar (Table 2: A1, Hungary vs. three areas in Germany). Closely related to this group, a large cluster of 32 isolates was detected containing isolates from...
Figure 3. MALDI-TOF MS dendrogram of *E. coli* and *Riemerella anatipestifer* reference data from the Bruker database and *R. anatipestifer* serotype 14 strains (MSP: master spectra)

MALDI-TOF MS-Dendrogramm der *E. coli*- und *R. anatipestifer*-Referenzdaten der Bruker Datenbank sowie der Serotyp 14 *R. anatipestifer*-Isolate (MSP, Hauptspektrum)

Figure 4. MALDI-TOF MS dendrogram of *Riemerella anatipestifer* serotype 14 isolates (strain number, ST: serotype, geographic origin, isolated from chicken unless mentioned otherwise. NW: Northwest, NE: Northeast)

Conclusions

Vaccination programs based on autogenous vaccines produced from relevant inactivated bacteria will remain the most suitable method for the prevention of *Riemerella* in ducks. The highly variable and dynamic evolution and distribution of *R. anatipestifer* field strains requires intensive monitoring and molecular subtyping of isolated strains even belonging to the same serotype. MALDI-TOF is a rapidly developing method and will contribute to a better understanding of the proteome of *R. anatipestifer* isolates and identifies potentially emerging clones of *R. anatipestifer*. Such results are of particular value for the production of efficacious autogenous vaccines.

Key words

Duck, *Riemerella anatipestifer*, serotype 14, autogenous vaccine, PFGE, MALDI-TOF MS

Zusammenfassung

Zur molekularen Typisierung von Feldstämmen von *Riemerella anatipestifer* Serotyp 14, einem Krankheitserreger mit zunehmender Bedeutung für Enten

Stichworte

Ente, Riemerella anatipestifer, Serotyp 14, bestands-
besonders spezifischer Impfstoff, PFGE, MALDI-TOF MS

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tion time-of-flight mass spectrometry evaluated by mul-

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