Characterization of Virulence Plasmids and Serotyping of *Rhodococcus equi* Isolates from Submaxillary Lymph Nodes of Pigs in Hungary

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The plasmid types and serotypes of 164 *Rhodococcus equi* strains obtained from submaxillary lymph nodes of swine from different piggeries in 28 villages and towns located throughout the country were examined. The strains were tested by PCR for the presence of 15- to 17-kDa virulence-associated protein antigen (VapA) and 20-kDa virulence-associated protein antigen (VapB) genes. Plasmid DNAs were isolated and analyzed by digestion with restriction endonucleases to estimate size and compare their polymorphism characteristics. None of the 164 isolates contained the *vapA* gene, and 44 (26.8%) isolates were positive for the *vapB* gene, showing a product of the expected 827-bp size in the PCR amplification. The 44 isolates of intermediate virulence contained virulence plasmids that were identified as types 1 (3 isolates), 4 (1 isolate), 5 (36 isolates), 6 (1 isolate), and 7 (2 isolates) and as a new variant (1 isolate). On the basis of restriction digestion patterns of plasmid DNAs, we tentatively designated the variant as type 17. Use of the serotyping method of Prescott showed that 110 (67.1%) out of the 164 isolates were typeable and that serotype 2 predominated (83 isolates [50.6%]), followed by serotype 1 (26 strains [15.9%]). Only one isolate belonged to serotype 3. A total of 54 (32.9%) isolates were untypeable in Prescott's system. The prevalence of *R. equi* strains of intermediate virulence among the isolates that came from the submaxillary lymph nodes of swine in Hungary was lower than that seen with isolates obtained elsewhere.

*R. equi* is an aerobic, gram-positive coccobacillus which frequently causes chronic suppurative bronchopneumonia with abscesses, lymphadenitis, and ulcerative enteritis in foals less than 6 months old (3, 14). In addition to its virulence for foals, *R. equi* seems also to be an important pathogen to immunocompromised humans, such as organ transplant and AIDS patients (21). *R. equi* is also common in the submaxillary lymph nodes of pigs (2, 8, 15, 25, 27). Katsumi and others (8) isolated *R. equi* from 45.6% of the submaxillary lymph nodes of swine with lesions and from 9.4% of lymph nodes of swine without lesions. Takai and colleagues (19) described a 3.1% isolation rate on the basis of examination of 1,832 submaxillary lymph nodes collected from swine.

Recently, the discovery of virulence-associated antigens and virulence plasmids has allowed classification of the virulence of *R. equi* strains (17, 24). At least three virulence levels of *R. equi* have been identified: virulence, intermediate virulence, and avirulence. Virulent *R. equi* is characterized by the presence of virulence-associated 15- to 17-kDa antigens (VapA), virulence plasmid DNAs of 85 to 90 kb, and suppurative pneumonia in foals (murine 50% lethal dose [LD50] = 106 cells). At this moment, there are at least 11 slightly different virulence plasmids in virulent isolates (26). *R. equi* strains of intermediate virulence are identified by the presence of a virulence-associated 20-kDa antigen (VapB), and virulence plasmids are found in the submaxillary lymph nodes of pigs (murine LD50 = 107 cells). Intermediately virulent strains contained one of 16 distinct plasmids of 79 to 100 kb found in human and pig isolates that were associated with the expression of the 20-kDa antigen (26). In comparison, avirulent *R. equi* contains neither virulence-associated antigens nor plasmid DNA (murine LD50 > 106 cells) (17). The majority of *R. equi* isolates from patients with AIDS are virulent (VapA+) or of intermediate virulence (VapB+), whereas most isolates from immunocompromised patients without AIDS were avirulent (21).

More recently, we demonstrated that five of the seven clinical isolates of *R. equi* from immunocompromised patients expressed VapB and that they were of intermediate virulence and revealed that these human isolates contained a 95-kb type 5 plasmid which was also seen in the pig isolates in Hungary (10). The route of infection in human cases is not clear. The purpose of this study was to isolate virulent *R. equi* strains from submaxillary lymph nodes of swine in Hungary, to determine the genotypic diversity of virulence-associated plasmids found in them, and to examine the serotypes of the isolates with the aim of finding additional data to characterize the epidemiological relationship between human *R. equi* infections and pigs carrying *R. equi* in the submaxillary lymph nodes.

Serotyping is a reliable method for examining *R. equi* strains. There are two systems for serotyping *R. equi*. Prescott de-
scribed seven serotypes by the use of an agar gel immunodiffusion (AGID) test (13), while Nakazawa et al. differentiated 27 serotypes with a slide agglutination test (12). Beside common antigens, several different ones have been found to react in the above-named tests; the results of the two serotyping systems do not completely agree (9). When the Prescott system is used, serotype 2 is the predominating serotype of \textit{R. equi} isolated from submaxillary lymph nodes of swine (8, 9, 12).

\section*{MATERIALS AND METHODS}

\subsection*{Source of isolates.} A total of 1,173 submaxillary lymph nodes without macroscopic lesions were collected in July of 2002 in a slaughterhouse in northeast Hungary. The sampled animals were kept in different piggeries in 28 villages and towns in Hungary. The sampled animals were kept in different piggeries in 28 villages and towns in Hungary.

\subsection*{Plasmid DNAs of the 16 newly identified in this study. Plasmid DNAs of the 16 representive types and of the 1 new type digested with EcoRI and EcoT22I digestion patterns, and lane 17 represents the plasmid type newly identified in this study. Plasmid DNAs of the 16 representative types and of the 1 new type digested with EcoRI and EcoT22I were examined by Southern analysis with PCR probes. The PCR products labeled with digoxigenin-11-dUTP hybridized with one of the fragments of each plasmid DNA (data not shown). From these results, we tentatively designated the new plasmid type as type 17.

\subsection*{DISCUSSION} 

The present study demonstrated that the presence of \textit{R. equi} is widespread (on average, in 14\% of pigs) in the submaxillary lymph nodes of healthy pigs in Hungary. \textit{R. equi} strains were isolated from 164 submaxillary lymph nodes of pigs of different piggeries in 28 villages and towns located throughout the country, and the majority (73.2\%) of \textit{R. equi} strains from pigs were avirulent (i.e., did not carry virulence plasmids), 26.8\% of the isolates were intermediately virulent, and none of the isolates represented virulent (vapA-positive) \textit{R. equi}. The finding of the prevalence of \textit{R. equi} of intermediate virulence in Hungarian

\section*{RESULTS} 

Out of 1,173 submaxillary lymph nodes collected from pigs, 164 \textit{R. equi} strains were isolated from 164 \textit{R. equi}-positive cultures. The rate of isolation was found to be 14.0\%, but there were differences among piggeries (range, 6.1 to 20.4\%). None of the 164 isolates analyzed by PCR contained \textit{vapA} gene. A total of 44 (26.8\%) strains were positive for the \textit{vapB} gene, showing a product of the expected 827-bp size in the PCR amplification. The 44 isolates of intermediate virulence (vapB positive) were then tested for the presence of virulence plasmids and analyzed by restriction enzyme digestion with endonucleases EcoRI and EcoT22I. Of the 44 isolates of intermediate virulence, 36 isolates contained a type 5 plasmid 95 kb in size, 3 contained a type 1 plasmid, 2 contained a type 7 plasmid, 1 contained a type 4 plasmid, and 1 contained a type 6 plasmid. The remaining one had unique restriction cleavage patterns and did not match any of the 16 previously reported EcoRI and EcoT22I digestion patterns for \textit{R. equi}. Lanes 1 to 16 in Fig. 1 represent the 16 previously reported EcoRI and EcoT22I digestion patterns, and lane 17 represents the plasmid type newly identified in this study. Plasmid DNAs of the 16 representative types and of the 1 new type digested with EcoRI and EcoT22I were examined by Southern analysis with PCR probes. The PCR products labeled with digoxigenin-11-dUTP hybridized with one of the fragments of each plasmid DNA (data not shown). From these results, we tentatively designated the new plasmid type as type 17.

When the method of Prescott was used, 28 (63.6\%) of the 44 intermediately virulent isolates and 82 (68.3\%) of the 120 avirulent isolates were typeable. By the same method, 54.5\% of the intermediately virulent and 49.2\% of the avirulent isolates belonged to serotype 2 and 15.9\% of the strains were of serotype 1. Only one isolate belonged to serotype 3. A total of 54 (32.9\%) strains were not typeable in Prescott’s system (Table 1).
FIG. 1. EcoRI (A) and EcoT22I (B) restriction fragments of the 17 plasmid types of *R. equi* isolates of intermediate virulence. Lanes 1, strain A2 (plasmid type 1); lanes 2, strain S2 (plasmid type 2); lanes 3, strain S3 (plasmid type 3); lanes 4, strain S4 (plasmid type 4); lanes 5, strain A5 (plasmid type 5); lanes 6, strain S6 (plasmid type 6); lanes 7, strain S7 (plasmid type 7); lanes 8, strain S8 (plasmid type 8); lanes 9, strain S9 (plasmid type 9); lanes 10, strain A11 (plasmid type 10); lanes 11, strain A43 (plasmid type 11); lanes 12, strain 70 (plasmid type 12); lanes 13, strain H3 (plasmid type 13); lanes 14, strain H25 (plasmid type 14); lanes 15, strain H43 (plasmid type 15); lanes 16, strain H66 (plasmid type 16); lanes 17, strain 316 (new plasmid type 17). The markers (lanes M) are HindIII digestion products of bacteriophage lambda DNA.
pigs was quite different from our previous finding that 368 (93.9%) of 392 isolates from the submaxillary lymph nodes of Japanese pigs were intermediately virulent, 2 (0.5%) of the isolates were virulent, and the remaining 22 (5.6%) were avirulent; however, the isolation rate in that study was lower (19). A difference between the methods of breeding pigs in Hungary (pigs were kept in natural environment) and in Japan (pigs were kept in isolated large-scale farms) may be the reason for the differences in the isolation rates and in the levels of prevalence of \( R. \text{equi} \) of intermediate virulence in pig isolates between Hungary and Japan. It might be interesting to investigate the prevalence of virulent \( R. \text{equi} \) in soil isolates from environment of piggeries in Hungary. The pathological significance of \( R. \text{equi} \) strains in the submaxillary lymph nodes of pigs cannot be judged, since the strains were isolated from lymph nodes without lesions.

\( R. \text{equi} \) is an emerging pathogen of humans, particularly for those with a compromised immune system (14, 21). Our previous study showed that five of the seven clinical isolates of \( R. \text{equi} \) from immunocompromised patients expressed VapB which contained a 95-kb type 5 plasmid (10). In the present study, the same plasmid type was found in the majority (81.9%) of the 44 pig isolates and the same serotype (serotype 2) was also found in 54.5% (24 of 44) of the intermediately virulent isolates. These results confirm the presumption that there is an epidemiological relationship between human \( R. \text{equi} \) infections and the presence of \( R. \text{equi} \) of intermediate virulence in submaxillary lymph nodes of pigs (19, 26).

<table>
<thead>
<tr>
<th>Pig isolate</th>
<th>No. of isolates</th>
<th>No. of isolates that were*&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avirulent</td>
<td>120</td>
<td>23</td>
</tr>
<tr>
<td>Intermediate virulent</td>
<td>44</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>164</td>
<td>26 (15.9)</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentages of the total number of isolates. NT, nontypeable.

The dominance of serotype 2 among the \( R. \text{equi} \) strains isolated from the submaxillary lymph nodes was shown in our examination. These results agree with the data in the literature (8, 9, 11); however, Prescott found serotype 1 to be the pre-dominating serotype among isolates from swine (13). A similar dominance of serotype 2 among \( R. \text{equi} \) strains isolated from humans was described by several authors (4, 9) and shows an epidemiological connection between pigs and humans.

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