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ORIGINAL ARTICLE

Virulence plasmids in clinical isolates of *Rhodococcus equi* from sick foals in the Netherlands

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Significance and Impact of the Study *Rhodococcus equi* is a bacterial pathogen well-known to cause pyogranulomatous pneumonia and lung abscesses in foals aged less than 6 months. This study showed that the virulent *R. equi* strains harbouring a virulence plasmid of 85-kb type I or 87-kb type I, which have been detected in clinical isolates from five European countries, are widespread in the Netherlands, and a new variant of pVAPA 52 kb was identified. This is the first report of plasmid types of clinical *R. equi* isolates in the Netherlands.

Keywords
foals, Netherlands, pVAPA, *Rhodococcus equi*, virulence plasmids.

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Abstract

Clinical samples from 123 foals with suspected rhodococcosis submitted to the Veterinary Microbiological Diagnostic Centre of the Faculty of Veterinary Medicine between 1993 and 2006 were tested for the presence of the virulence gene *vapA*. Of the 123 samples, 120 were *vapA*-positive and 3 *vapA*-negative *Rhodococcus equi* were isolated. The 120 *vapA*-positive *R. equi* were isolated from 70 tracheal wash, 19 lung tissues, 7 lymph nodes, 6 synovial fluids, 13 abscesses or pus and single isolates from the uterus, gut, cerebrospinal fluid, abdomen fluid and faeces. Of the 120 isolates, 46 were from Dutch warmblood horses, 23 from Friesian horses, 14 from Trotters, 4 from Holsteiners, 3 from Arab breed, 2 from ponies, 1 from a Welsh pony and 27 from undefined breed horses. Using plasmid profile analysis of the 120 isolates, 117 isolates contained the 85-kb type I plasmid, 2 contained the 87-kb type I plasmid and 1 contained the novel 52-kb non-mobilizable virulence plasmid reported recently. These results showed that the virulent *R. equi* strains harbouring a virulence plasmid of 85-kb type I or 87-kb type I, which have been detected in clinical isolates from five European countries, are widespread in the Netherlands. This is the first report of plasmid types of clinical *R. equi* isolates in the Netherlands.

Introduction

Rhodococcus equi is a well-known bacterial pathogen first described in a Swedish foal in 1923 as the causative agent of pyogranulomatous pneumonia and lung abscesses in foals aged less than 6 months (Magnusson 1923). *Rhodococcus equi* is widespread, inhabits the soil and colonizes the intestine of grazing animals and omnivores, and more recently, it was isolated from the earthworm's gastrointestinal contents (Takai *et al.* 2022). *Rhodococcus equi* also infects other mammals, including pigs, cattle, goats, cats, dogs and humans (Vázquez-Boland and Meijer 2019).

The pathogenicity of *R. equi* is associated with the presence of a virulence plasmid encoding a family of 'virulence-associated proteins' (Vaps) (Takai *et al.* 2000). Two different *R. equi* circular virulence plasmid variants were initially characterized: the VapA-encoding pVAPA, found in virulent isolates of equine origin, and the VapB-encoding pVAPB, found in isolates exhibiting intermediate virulence in mice, recovered from submaxillary lymph nodes of slaughtered pigs and human clinical specimens

(Takai *et al.* 1991a, 1991b, 1995). Recently, the VapN-encoding linear virulence plasmid, pVAPN, was found in isolates from bovids and human clinical specimens (Valero-Rello *et al.* 2015; Takai *et al.* 2020). The three virulence plasmids are host-specific, with pVAPA associated with horses, pVAPB with pigs and pVAPN with cattle and goats (ruminants) (MacArthur *et al.* 2017).

The diversity of virulence plasmids (pVAPA) by restriction enzyme digestion patterns has been evaluated (Takai *et al.* 1999, 2001a, 2001b, 2001c, 2003; Ribeiro *et al.* 2005; Son *et al.* 2006), and recently, Witkowski *et al.* (2017) reported that one isolate from a Polish foal had a peculiar restriction cleavage pattern and the second fragment of *Eco*RI digests of this plasmid DNA was approximately 2600 bases smaller than that of the 85-kb type I. They designated this new plasmid variant as the '85-kb type V'. To date, 13 closely related plasmid types (85-kb types I, II, III, IV and V, 87-kb types I, II and III and 90-kb types I, II, III, IV and V) have been found in virulent *R. equi*. More recently, Suzuki *et al.* (2022) have reported the 14th new variant of pVAPA_U19, 52-kb in an isolate from the tracheal wash of a foal in the Netherlands.

Rhodococcus equi infection in foals has been recognized in the Netherlands but without any information about the molecular characteristics of *R. equi* isolates. This study characterizes the types of virulence plasmids in clinical isolates cultured at the Veterinary Microbiological Diagnostic Centre of Faculty of Veterinary Medicine, Utrecht University in the Netherlands between 1993 and 2006.

Results and discussion

At the Veterinary Microbiological Diagnostic Centre (VMDC) of Faculty of Veterinary Medicine, Utrecht University in the Netherlands, clinical samples from foals suspected of rhodococcosis were admitted between 1993 and 2006, and *R. equi* was isolated from the 123 samples from 123 distinct foals. The isolates were stored in the strain collection of the VMDC. Of the 123 isolates, 120 were *vapA*-positive and 3 were negative (Table 1). *vapA*-positive *R. equi* was isolated from 70 tracheal wash, 19 lung tissues, 7 lymph nodes, 6 synovial fluids, 13 abscesses or pus and single isolates from the uterus, gut cerebrospinal fluid, abdomen fluid and faeces. Of the 120 isolates, 46 were from Dutch warmblood horses, 23 from Friesian horses, 14 from Trotters, 4 from Holsteiners, 3 from Arab breed, 2 from ponies, 1 from a Welsh pony and from 27 from horses with a non-specified breed. Of the 120 isolates, 117 (97·5%) contained the 85-kb type I plasmid, 2 (1·7%) contained the 87-type I plasmid and 1 (0·8%) contained a new variant, a 52-kb virulence plasmid, which was recently reported as the smallest non-self-transmissible virulence plasmid in *R. equi*. This plasmid is similar to the 85-kb type I plasmid in terms of pathogenicity and plasmid maintenance, but it lacks almost all genes related to mobility (MOB) genes, which allow conjugative DNA processing, and mating pair formation (MPF) genes, which are a form of the type IV secretion system and provide the mating channel (Suzuki *et al.* 2022).

The distribution of virulence plasmid types in the 120 isolates by plasmid profile analysis is shown in Tables 1–3 by isolation year, source and horse-breeds. Isolates carrying the 85-kb type I plasmid were found throughout the Netherlands, irrespective of isolation year, source or breed of the horse.

Table 1 Distribution of virulence plasmid types in *Rhodococcus equi* isolates from 123 foals in the Netherlands by year

Year	No. of isolates	Presence of *vapA*	Virulence plasmid types			Avirulent
			85-I	87-I	52 kb	
1993	4	4	4			
1994	4	4	4			
1995	3	3	3			
1996	7	7	7			
1997	5	5	4		1	
1998	12	12	12			
1999	14	14	14			
2000	16	16	16			
2001	9	8	7	1		1
2002	8	8	8			
2003	9	9	9			
2004	10	10	10			
2005	10	10	10			
2006	12	10	9	1		2
Total	123	120	117	2	1	3

Table 2 Distribution of virulence plasmid types in *vapA*-positive isolates by source

Source	No. of isolates	Virulence plasmid types		
		85-I	87-I	52 kb
Tracheal wash	70	67	2	1
Lung tissue	20	20		
Lymph nodes	7	7		
Synovial fluid	6	6		
Abscess or pus	13	13		
Uterus	1	1		
Gut	1	1		
Cerebrospinal fluid	1	1		
Fluid from abdomen	1	1		
Faeces	1	1		
Total	120	117	2	1

Table 3 Distribution of virulence plasmid types in *vapA*-positive isolates by horse breeds

Horse breeds	No. of isolates	Virulence plasmid types		
		85-I	87-I	52 kb
Dutch warmblood	46	45	1	0
Friesian horse	23	22	0	1
Trotter	14	14	0	0
Arab	3	3	1	0
Holsteiner	4	4	0	0
Pony	2	2	0	0
Welsh pony	1	1	0	0
Unknown	27	27	0	0
Total	120	117	2	1

In this study, the majority (97·5%) of the clinical isolates of *R. equi* from foals in the Netherlands contain pVAPA 85-kb type I, and 2 (1·7%) contained 87-kb type I, similar to the plasmid profiles of *vapA-R. equi* in the other European countries and Americas. It is worth noting here that the new pVAPA 52-kb variant was found in the isolate from the tracheal wash of a Friesian horse in 1997, and it was never found elsewhere during the years.

As reported recently (Suzuki *et al.* 2022), the restriction fragment length polymorphism of pVAPA_U19 digested using *Eco*RI was similar to that of pREAT701 (85-kb type I) harboured by *R. equi* ATCC33701. Whole-genome sequencing indicated that pVAPA_U19 was 51 684 bp in length and that the *vapA* pathogenicity island region and the replication/participation were almost identical to those in pREAT701. Additionally, pVAPA_U19 does not vary significantly from the previously reported pVAPA in terms of virulence and plasmid replication and maintenance. Still, it is a non-mobilizable plasmid unable to induce conjugation due to the absence of genes related to MOB and MPF (Suzuki *et al.* 2022). This new variant was tentatively designated as the 52-kb plasmid. Future research on the smallest new variant in the breeding environment soil on the stable where this strain has been found is desirable.

The movement of horses might be associated with the spread of infectious diseases. The plasmid profile analysis of virulence plasmids has shown their geographic distribution globally (Witkowski *et al.* 2017). The diversity of pVAPA using restriction enzyme digestion patterns has been studied in European countries, such as France (Rahal *et al.* 1999; Duquesne *et al.* 2010), Germany (Venner *et al.* 2007), Poland (Kalinowski *et al.* 2016; Witkowski *et al.* 2017), Hungary (Makrai *et al.* 2006) and Turkey (Ozgur *et al.* 2000). 85-kb type I and 87-kb type I plasmids were found in the Netherlands as well as the other five European countries. The 85-kb type II plasmid has been found only in French isolates (Rahal *et al.* 1999). The 85-kb type III and type IV plasmids were found only in isolates from Texas (Takai *et al.* 2001a), and the 87-kb type II and 90-kb type I–IV plasmids have been found only in clinical and environmental isolates from Japan (Takai *et al.* 2001b, 2001c). Two plasmid types, 90-kb type II and V plasmids, are found in isolates from Jeju Island, Korea (Takai *et al.* 2003). To better understand the basis of the genetic diversity between *vapA*-carrying virulence-associated plasmids, sequencing and molecular analysis of the 14 types of virulence plasmids are in progress.

Materials and methods

Clinical samples

Between 1993 and 2006, 123 clinical samples from sick foals from 109 foals located in the Netherlands were admitted to the Veterinary Microbiological Diagnostic Centre of Faculty of Veterinary Medicine, Utrecht University in the Netherlands were found positive for *R. equi* by culture. Suspected bacterial colonies were subcultured to get pure culture. Identification of strains as *R. equi*, including the U19 strain previously reported by Suzuki *et al.* (2022), was conducted on colony morphology, Gram staining, biochemical profiling using API Coryne test (BioMerieux, Marcy l'Etoile, France; cat. no 20900). Bacterial strains identified as virulent *R. equi* were stored frozen in 20% glycerol at −70°C.

PCR and plasmid DNA analysis

The presence of *R. equi* genes, *cox* and *vapA*, were determined using PCR (Ladrón *et al.* 2003). Plasmid DNA was isolated from *R. equi* using the alkaline lysis method with some modifications, as previously described (Takai *et al.* 1991b). Plasmid DNAs were analysed by digestion with restriction endonucleases *Bam*HI, *Eco*RI, *Eco*T22I and *Hin*dIII for detailed comparison and estimation of the plasmid size (Takai *et al.* 1999). Samples of the plasmid preparations were separated in 0·7% or 1·0% agarose gels at about 5-V cm^{-1} for 2 h. The bacterial strains used as reference strains in this study were *R. equi* ATCC 33701 (85 kb-type I), 96E35 (85 kb-type II), T47-2 (85 kb-type III), T43 (85 kb-type IV), 222 (87 kb-type I), 96B6 (87 kb-type II) and Brazil 40 (87 kb-type III) (Takai *et al.* 1999, 2001b, 2001c; Ribeiro *et al.* 2005).

Author contributions

All authors contributed to conception, design and analysis of data. Shinji Takai, Masaki Ohashi, Yasunori Suzuki, Yukako Sasaki, Tsutomu Kakuda, Els M. Broens, Jaap A. Wagenaar and Engeline van Duijkeren conducted the experiments. Shinji Takai, Yasunori Suzuki and Engeline van Duijkeren contributed to drafting or critically revising the manuscript, and approval of the final submitted version.

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Conflicts of Interest

All the authors of the present manuscript have no conflict of interest to declare.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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