MOLECULAR CHARACTERIZATION AND PHENOTYPING OF *PASTEURELLA* FROM SWISS RABBITS

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ABSTRACT

Several bacteria belonging to the family *Pasteurellaceae* might act as pathogens in rabbits. In particular, *Pasteurella multocida* is considered to be an important one and outbreaks caused by this species result in considerable economic losses. However, proper identification of *P. multocida* at the species and even more so at the subspecies level is often not very precise. Moreover, *Pasteurellaceae* species isolated from rabbits are poorly characterized. We therefore phenotypically and genotypically analyzed 228 isolates from different rabbit populations including a breeding and fattening organization with group management as well as isolates from single post-mortem cases with pasteurellosis using biochemical reactions and repetitive extragenic palindromic (REP)-PCR. Furthermore, 41 samples were selected for phylogenetic analysis of 16S rRNA gene. REP-PCR identification and phylogenetic analyses correlated well and appeared to be distinct molecular methods for identification of rabbit isolates. Phenotyping, however, diverged from molecular recognition, indicating the problematic conventional diagnosis of these strains. The fermentation of sorbitol and trehalose appeared to be imprecise indicators for *P. multocida* subspecies classification. However, according to REP-PCR and sequencing results, 82% of the isolates were characterized as *P. multocida* ssp. *multocida*, 3% as *P. multocida* ssp. *septica*, 5% could be assigned to *P. multocida* and probably represented a new subspecies within this species. Further, 5% were assigned to *Pasteurella canis*. The other 5% represented a homogeneous group of unknown species belonged to the *Pasteurellaceae*. Samples obtained from post-mortem cases demonstrated a higher phenotypic and genetic heterogeneity than samples from group management rabbits.

Key words: *Pasteurella*, Rabbit, REP-PCR, 16S rRNA, Phenotype.

INTRODUCTION

Bacterial diseases, often induced by *Pasteurella multocida*, are predominant causes of death in rabbits. Non-infected and resistant animals, chronic healthy carriers, animals with local infections (rhinitis, otitis media), pneumonia and septicemia can be distinguished (Deeb *et al.,* 1990). In contrast to *P. multocida*, little is known about the occurrence and importance of other members of the family *Pasteurellaceae* in rabbits.

The current identification of *Pasteurellaceae*, mainly based on a complex phenotypic characterization, is time consuming and sometimes imprecise. Moreover, discrimination between subspecies of *P. multocida*, namely as *P. multocida* ssp. *multocida*, *P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida* based on sorbitol, trehalose, as well as dulcitol fermentation reactions is ambiguous (Blackall *et al.,* 1997). A more feasible tool for this purpose is the DNA-sequence-based identification. The comparative sequence analysis of the ribosomal RNA coding gene (16S rRNA) has been successfully used for identification and for clarifying the phylogenetic relationship within the family *Pasteurellaceae* as well as on the *P. multocida* subspecies level (Christensen *et al.,* 2004). Repetitive extragenic palindromic (REP)-PCR is a molecular technique, which enables analysis of the distribution of noncoding REP elements in the entire bacterial genome.

In the present study, we investigated the phenotypic and genetic diversity within strains belonging to the *Pasteurellaceae* family obtained from rabbits by biochemical analysis, REP-PCR and 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Sources of isolates

One hundred and twenty-three isolates from the nares or sinus of slaughtered group management rabbits were gathered. The animals came from different breeding and fattening farms of one Swiss rabbit meat organization. One hundred and five strains were isolated from altered organs of post-mortem cases with pasteurellosis, sent to our department by various rabbit owners.

Phenotyping

All 228 isolates were inoculated on 5% sheep blood agar and incubated under aerobic conditions at 37°C for 24 hours. The biochemical characterization was carried out as described previously (Mutters *et al.,* 1985).

Molecular characterization by REP-PCR

Fragments between the noncoding repetitive extragenic palindromic elements present in rabbit *Pasteurellaceae* isolates were generated in REP-PCR using following degenerated primers REP1R-IDt: 5'–NNNNGCNGCNGTAGNCCG–3' and REP2-IDt: 5'-NCGNCTTATCNGGCCTAC-3' (Townsend *et al.,* 1997). The PCR mixture of a 50 µl final volume contained 1 x PCR buffer with 4 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (TaqBead™ Hot Start Polymerase, Promega, WI, USA), 200 µM of each of the four dNTPs (Promega), 50 pmol of each primer and 2.5 µl in advance prepared bacterial cell lysate. In short, 3 to 4 bacterial colonies from blood agar plates were transferred in 50 µl nuclease-free water (Promega) and boiled in a heating block at 100°C for 10 min. After centrifugation at 10000 x g for 5 min, the supernatant was collected and stored at 4°C until use. REP-PCR was run in a DNA 2720 Thermal Cycler (Applied Biosystems, CA, USA) with an initial denaturation step at 95°C for 7 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min and extension at 65°C for 8 min. The PCR was terminated with a final extension at 65°C for 16 min (Rademaker and Bruijn, 1997). The amplified fragments were separated on a 1.5% agarose gel with ethidium bromide in 1 x TAE at 70 V for 240 min.

Sequence analysis of the 16S rRNA gene

Based on REP-PCR results and phenotyping, 41 *Pasteurella* rabbit isolates were selected for sequencing and phylogenetic analysis of the 16S rRNA genes. Sequencing products were run on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems) followed by editing using the SEQUENCHER software (GeneCodes, MI, USA). The sequences obtained were cross-compared against GenBank (Benson *et al*., 2000) and SmartGene (Simmon *et al.,* 2006) databases. Phylogenetic analysis of the proof-read sequences and selected GenBank entries, representing each described genus within the *Pasteurellaceae* family, was performed using neighbor-joining tree building with the BioNumerics program version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). All sequences were submitted to GenBank (National Center for Biotechnology Information, Bethesda, MD) under the accession numbers ranging from EF579813 to EF579894.

RESULTS

Phenotyping

Based on the biochemical activity of the strains a total of twelve diverse biochemical types (BT) could be recognized (Table 1).

Table 1: Comparison between REP-PCR and biochemical differentiation of *Pasteurella* isolates from Swiss rabbits

		Pasteurella multocida										*P. canis*	*P.* sp.?
		ssp. *multocida*								*septica*	*gallicida*	*biotype 1*	
		1	2	3	4	5	6	7	8	9	10	11	12
Biochemical test	Indole	+	+	+	+	+	-	+	-	+	+	+	-
	Dulcitol	-	-	-	-	-	-	-	-	-	+	-	+
	Sorbitol	+	+	+	+	+	+	-	-	-	+	-	-
	Mannitol	+	+	+	+	+	+	+	+	+	+	-	-
	Trehalose	+	-	+	-	+	-	-	-	+	-	+	+
	Maltose	-	-	-	-	+	-	-	-	-	-	-	+
	ODC	+	+	-	-	+	+	+	+	+	+	+	-
REP-PCR types	I	-	*76* **34**	**1**	**3**	-	**3**	*36* **32**	**2**	-	-	-	-
	II	-	**5**	-	*1* **5**	-	-	-	-	-	-	-	-
	III	-	-	-	-	-	-	-	-	-	**1**	-	-
	IV	-	-	-	-	-	-	-	-	**3**	-	-	-
	V	**2**	-	-	-	-	-	-	-	**1**	-	-	-
	VI	**1**	-	-	-	-	-	-	-	-	-	-	-
	VII	-	-	-	-	-	-	-	-	-	-	*10* **1**	-
	VIII	-	**1**	-	-	-	**1**	-	-	-	-	-	**8**
	IX	-	-	-	-	**1**	-	-	-	-	-	-	-

1-12: biochemical types
+: positive reaction; -: negative reaction for 14 days
?: not assignable to any recognized Pasteurella species/subspecies
in italic are number of isolates from group management and in bold are number of isolates from clinical cases

Molecular characterization by REP-PCR

Application of REP-PCR for all investigated strains resulted in nine different patterns. Isolates obtained from group management rabbits in particular belonged to the REP-PCR types I, one strain showed pattern typical for the type II, and ten were assigned as the type VII. Isolates from post-mortem cases were much more heterogeneous covering each of the nine REP-PCR types.
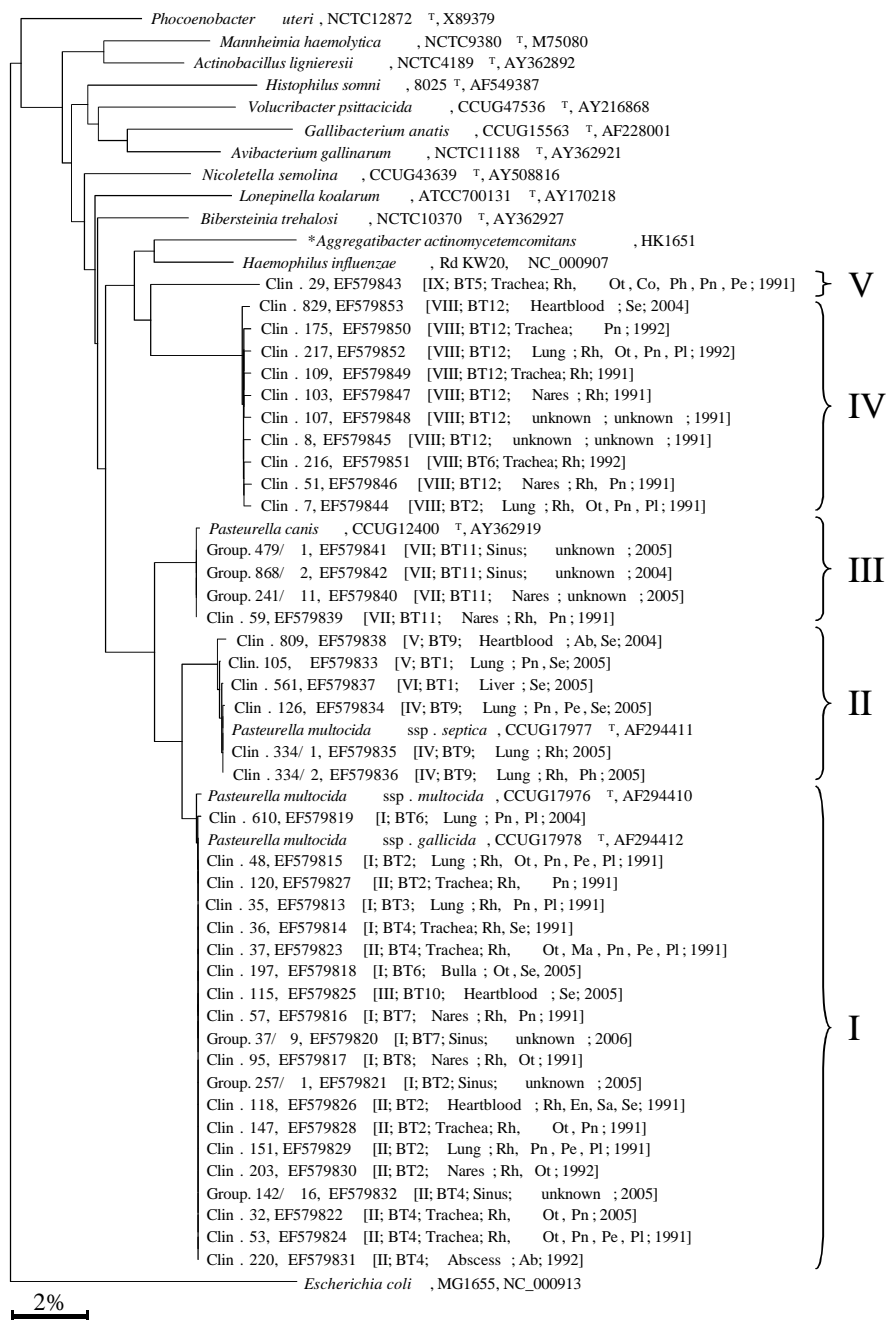
Sequence analysis of the 16S rRNA gene

After truncation of primer sequences, the 16S rRNA gene sequencing in most cases resulted in a fragment of 1364 bp, however, ten samples gave a fragment of 1356 bp. The phylogenetic relationships of the 16S rRNA gene sequences of 41 isolates representing all observed phenotypes and REP-PCR patterns are illustrated in Figure 1. In the 16S rRNA-based tree the strains were grouped in five clusters.

DISCUSSION

This is the first extended study of a large collection of *Pasteurella* isolates from rabbits. The conventional phenotyping demonstrated a high heterogeneity, resulting in the recognition of 12 biochemical types. This variability - in particular within the species *Pasteurella multocida* - is well

known (Biberstein *et al.,* 1991). In this study 30% of all 228 isolates could not be identified correctly by phenotyping as the differentiation between sorbitol negative variants of *P. multocida* ssp. *multocida* and trehalose negative variants of *P. multocida* ssp. *septica* was impossible.

Figure 1: 16S rRNA sequence-based phlyogenetic tree of 41 representative rabbit isolates and selected from GenBank entries represented all currently known genera within the family *Pasteurellaceae*. The species names, strain numbers and accession numbers are listed and grouped in clusters I to V. *E. coli* was used as an outgroup. The scale bar represents percentage of sequence divergence

Strain acronyms: Clin. for clinical cases, Group. for group management, [REP-PCR type I-IX; biochemical types (BT) 1-12; Origin; Disease status: Rh=Rhinitis, Ot=Otitis media, Pn=Pneumonia, Pe=Pericarditis, Pl=Pleuritis, Co=Conjunctivitis, Ph=Phlegmone, Ab=Abscess, Me=Meningoencephalitis, Ma=Mastitis, En=Endometritis, Sa=Salpingitis, Se=Septicemia; Year of isolation]
*www.genome.ou.edu

The sequencing of the 16S rRNA gene leads to better taxonomic classification of *Pasteurella* and related bacteria. This method enabled the grouping of the 12 biochemical types of our study into 5 genetic clusters with 2 subspecies of *Pasteurella multocida* and demonstrated the ability to differentiate inter- and intra-subspecies variations. Furthermore, identical biochemical reactions of BT 2, BT 4 and BT 6, phenotypically identified as *P. multocida* ssp. *multocida* and variations of the such, clustered in two different phylogenetic clusters (I, IV).

REP-PCR is a distinct molecular method for the classification of *Pasteurella* strains from rabbits. REP-PCR was shown to be in high correlation with DNA-sequence-based identification. In this study the isolates clustered into 9 REP-PCR types compared to sequence analysis with 5 main phylogenetic groups. Certain REP-PCR bands seem to be distinct for a specific *Pasteurella* species or subspecies. All 9 genotypes were attributable to different *Pasteurella* species and subspecies, e.g. *P. canis* biotype 1, *P. multocida* ssp. *septica* and 2 separate groups, which could not be assigned to a known species. Therefore REP-PCR is a precise, reproducible and easy applicable method with an excellent discriminatory power for the inter- and intra-species genotyping of *Pasteurella* strains as was reported (Chen *et al.,* 2002). With 112 of 123 isolates (91%), a particular genotype (REP-PCR type I) of *P. multocida* ssp. *multocida* is widely spread throughout the Swiss population of group management rabbits. The top down practice with one main breeder delivering animals to all other farmers, led to the distribution of this particular strain to different rabbitries. The isolates from post-mortem cases showed a high heterogeneity. Only 75 of the 105 isolates (72%) were grouped as *P. multocida* ssp. *multocida*.
In conclusion, *P. multocida* ssp. *multocida* is the most common subspecies present in Swiss rabbits, but other *Pasteurella* subspecies have been found in clinical material. *P. multocida* ssp. *septica* strains represented a small number of the isolates (3%). In addition 11 isolates of a yet unknown *Pasteurella* group and one single isolate, which could not be assigned to a known species, were identified by sequence analysis and referred to as rabbit pathogen.

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