PCR (Polymerase Chain Reaction) is Superior to Culture and Serology in Detecting Haemophilus Infection in Rats and Guinea Pigs

by ***R. Boot*[1,*]*** ***& F.A.G. Reubsaet*[2]**

[1]Section of Laboratory Animal Microbiology Laboratory for Infectious Diseases and Screening, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

[2]Section of Bacterial Diagnostics, Laboratory for Infectious Diseases and Screening, National Institute of Public Health and the Environment, Bilthoven, The Netherlands

Summary

Based on partial sequencing of the 16S rRNA gene V-factor dependent *Pasteurellaceae* (*Haemophilus*), strains from rat and guinea pig were assigned to the Rodent cluster or the *Haemophilus parainfluenzae* complex.

PCRs for the detection of biotype Heyl or Jawetz [*P.*] *pneumotropica* detected none of the strains and only two *Haemophilus* strains assigned to the Rodent cluster respectively. All *Haemophilus* strains were positive by a PCR developed for detection of all *Pasteurellaceae* taxa.

The *Pasteurellaceae* PCR detected infection in all 76 rats and 40 guinea pigs from 3 and 6 colonies respectively reported to be free from *Pasteurellaceae* infection.

ELISAs, using two *Haemophilus* antigens and culture, detected infection with similar frequency but both methods were inferior to PCR.

The *Pasteurellaceae* PCR should be the new 'gold standard' for comparison of the sensitivity of other test methods for *Pasteurellaceae* infection in rodents.

Introduction

A PCR developed to detect all *Pasteurellaceae* taxa (*Bootz et al., 1998*) was found superior to PCRs for Heyl or Jawetz biotype [*P.*] *pneumotropica* in detecting growth factor independent rodent *Pasteurellaceae* (*Boot et al., 2009*).

Contemporary Specified Pathogen Free (SPF) colonies are however frequently infected by V-factor requiring *Pasteurellaceae* traditionally called *Haemophilus* spp (*Nicklas, 1989; Nicklas et al., 1993; Boot et al., 1998*).

**Correspondence:* R Boot
Retired from: Section of Laboratory Animal Microbiology, Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands
E-mail r.boot@hotmail.com

Based on genetic studies rodent *Pasteurellaceae* requiring V-factor have been assigned to the [*P.*] *pneumotropica* biotype Jawetz complex (*Ryll et al., 1968*) and to Bisgaards taxon 22 (*Hayashimoto et al., 2007*) which are both part of the Rodent cluster of the bacterial family (*Olsen et al., 2005*). Biochemically other rodent *Haemophilus* strains were found likely to belong to the *H. parainfluenzae* complex (*Nicklas et al., 1993*).

In the present study we found rat and guinea pig *Haemophilus* strains to belong to the Rodent cluster and the *H. parainfluenzae* complex, as based on partial 16S rDNA sequencing. The *Pasteurellaceae* PCR detected significantly more *Haemophilus* strains than PCRs for both [*P.*] *pneumotropica* biotypes and the assay appeared superior to culture and

[1] by convention [..] brackets around the genus name indicate misclassification.

serology in the detection of natural *Haemophilus* infection in rats and guinea pigs.

Materials and Methods

Bacterial strains

Bacterial strains examined comprised fifteen V-factor requiring *Pasteurellaceae* (*Haemophilus* sp) obtained in previous studies from rats (n= 9) (*Boot et al., 1994*) and guinea pigs (n= 6) (*Boot et al., 1998*). All bacterial strains were grown on 5% heated sheep blood agar (chocolate agar) (Oxoid, Haarlem, the Netherlands) incubated for 18 h at 37 °C under 7.5 % CO_2. Growth was harvested in 100 µl MQ (Sigma Chemical Co., St Louis Mi, USA) and heated for 10-15 min in a heating block. Lysates were frozen at - 86°C until testing by PCR.

Phylogenetic analysis

Part (about 457 nucleotides) of the gene coding for the 16S rRNA was sequenced of *Haemophilus* strains from rat (n= 9) and guinea pig (n= 6) (Table 1). The sequences were compared with sequences of

Table 1. API NH code, FAME profiles and homology with closest related *Pasteurellaceae* reference strains of V-factor dependent *Pasteurellaceae* strains (*Haemophilus* sp) from rats and guinea pigs.

Strain	Host	API NH code	FAME profile	% homology (accession nr)	% Homology (accession nr)
V6	Rat	n.t.	n.t.		98% [*Pasteurella*] sp. MCCM00235 (AF224300)
H12	Rat	7560	3	98% [*Haemophilus*] sp. V6 (AB279579)	96% "
H21	Rat	7766	1	100% "	98% "
H185	Rat	7766	1	99% "	98% "
H249	Guinea pig	7504	11	99% Bisgaard Taxon 22 (AY172726)	
H169	Rat	7172	7	99% "	
H143	Guinea pig	7766	5	99% "	
H216	Guinea pig	7766	9	99% "	
H184	Rat	7766	2	98% "	
H183	Rat	7520	1		97% [*H*]. *parainfluenzae* (EU083530)
H200	Guinea pig	7720	10		97% "
H180	Rat	7720	1		97% "
H35	Guinea pig	7720	2		97% "
H224	Guinea pig	7720	12		98% "
H98	Rat	7360	2		98% "
H181	Rat	7362	2		98% "

reference *Pasteurellaceae* from rodents deposited in Genbank. A 16S rDNA dendrogram was constructed using the neighbour joining method (Multi-way option AlignPlus 5.0 SciEd software (Durkam, USA)).

Polymerase chain reaction

DNA lysates of all bacteria were tested in duplicate using three primer sets, namely a set for *Pasteurellaceae* (*Bootz et al., 1998*), a set for Heyl biotype [*P.*] *pneumotropica* (*Kodjo et., 1998*) and a set for Jawetz biotype [*P.*] *pneumotrotropica* (*Wang et al., 1996*).

Primers were synthetized by Gibco BRL Life Technologies (Breda NL).

DNA amplification and detection of amplicons was performed as described (*Boot et al. 2009*)

Preparation of PCR mixtures, addition of template DNA and detection and analysis of PCR products were done in separate rooms to prevent intercurrent contamination of reaction mixtures by *Pasteurellaceae* DNA.

Phenotypic characterization

All strains were studied by the API NH biochemical profiling system and by cellular fatty acid profiling as described (*Boot, 2008*).

Animals

Rats and guinea pigs from commercial breeding and experimental colonies were examined. In some colonies *Haemophilus* spp infection had been previously detected by culture and serology.

Rats (n=76) comprised 8 inbred strains from 3 microbiological units. Guinea pigs (n=40) comprised 6 DH strains from 6 units (Tables 2 and 3). All breeding colonies were reported free from infection by *Pasteurellaceae.*

Animals were exsanguinated under KRA [Ketamine (Alfasan, Woerden NL) 90 mg/kg i.p., Rompun (Bayer AG, Leverkusen Germany) 10 mg/kg i.p., atropine (Vetinex Animal Health, Bladel NL) 0.05 mg / kg i.p.) anesthesia and examined for *Pasteurellaceae* infection by culture, antibody ELISA and by PCR.

Table 2. Detection of *Pasteurellaceae* infection in rats naturally infected by V-factor requiring species (*Haemophilus*)

Breeder	unit	strain	n =	Culture	ELISA*	PCR
A	1	WU	28	24	20	28
B	1	PVG	13	1	13	13
C	1	LEW	6	4	4	6
		WKY	6	4	6	6
		BN	6	4	6	6
		BD	5	2	4	5
		SHR	6	6	3	6
		F344	6	4	5	6
		n=	76	49	61	76
		% pos		64	80	100
negative control colony		n > 80		0	0	0

* combined results of testing using antigens of [*P.*] *pneumotropica* and *Haemophilus* strains H21 and H35

Table 3. Detection of *Pasteurellaceae* infection in guinea pigs naturally infected by V-factor requiring species (*Haemophilus* spp)

Breeder	unit	strain	n =	Culture	ELISA*	PCR
A	1	DH	15	3	3	15
	2	"	5	2	1	5
	3	"	5	3	4	5
B	1	"	5	5	4	5
C	1	"	5	4	1	5
	2	"	5	4	0	5
		n =	40	21	13	40
		% pos.		52.5	32.5	100
negative control colony		n > 80		0	0	0

* combined results of testing using antigens of *Haemophilus* strains H21 and H35

Rats and guinea pigs from our hysterectomy-derived barrier-maintained *Pasteurellaceae*-free breeding colonies submitted for routine health monitoring served as controls.

Sampling was carried out in a sterilized safety cabinet and strict precautions were taken to avoid cross-contamination between animals and samples.

Culture

Samples were taken by moistened cotton swabs from the pharynx and inoculated onto plain sheep blood agar and chocolate agar, both types made selective by the addition of 2 g/l clindamycin HCl. Agars were incubated for 48 hrs aerobically (SBA) or under 7.5 % CO_2. Characterization of *Pasteurellaceae,* notably *Haemophilus* spp, suspected growth was carried out as described (*Boot et al., 1994/5*).

Serology

Sera (1: 50 diluted) were tested by ELISA as described (*Boot et al., 1995*) using whole cell antigens of [*P.*] *pneumotropica* NCTC 8284 and *Haemophilus* strains H21 and H35. Immune sera (positive control) and sera from *Pasteurellaceae* uninfected animals (negative control) were simultaneously tested.

PCR

DNA in samples taken by swabs from the pharynx was extracted using the DNeasy Tissue Kit (Quiagen, Hilden Germany) according to the manufacturer's instructions. The PCR was carried out using Bootz's primers in a concentration of 5 pmoles (both). All reaction conditions were as described (*Boot et al., 2009*).

Lysate of [*P.*] *pneumotropica* NCTC 8284 (bacteria treated 15 min at 100 °C in MQ) was included as a positive control and MQ was included as a negative control in all runs. Amplicons were stored at 4 °C.

Statistical analysis

Differences in the number of *Haemophilus* strains detected by PCR were analysed by Fisher's exact test.

Differences in the number of positive animals were analysed by the Cochrans's Q test and McNemars test for dichotomous variables. The level of significance was set at $p < 0.05$. Calculations were performed using the Genstat statistical package run on a PC.

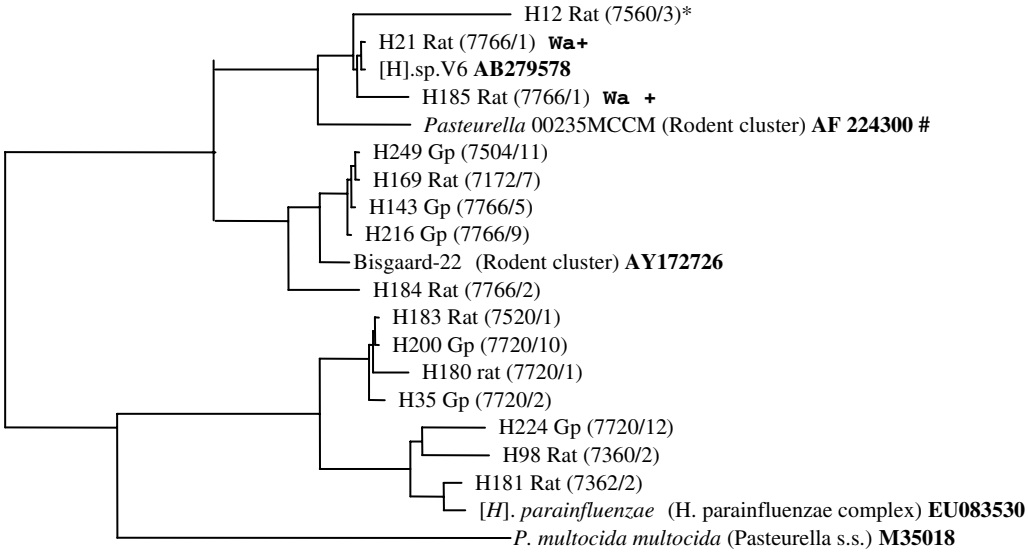

Figure 1. Unrooted neighbour joining tree based on partial 16S rDNA gene sequences showing the phylogenetic relationship of rat and guinea pig V-factor dependent *Pasteurellaceae* (*Haemophilus* sp) and complete 16S rDNA gene sequences of closest related *Pasteurellaceae* as deposited in Genbank.
* strain number, host species (API NH code/ FAME profile);
# Genbank accession number for 16S rDNA sequences of reference strains;
Wa+: reaction in PCR with Wangs primer set for biotype Jawetz [*P.*] *pneumotropica.*

Results

Bacterial strains

Phylogenetic analysis

Based on partial 16S rDNA sequencing (Fig. 1) our *Haemophilus* strains showed highest similarities with gene sequences of *Haemophilus* strain V6 (*Hayashimoto et al., 2007),* [*P.*] *pneumotropica*-like MCCM 00235 (*Olsen et al., 2005*), Bisgaards taxon 22 (*Christensen et al, 2003*) and *H. parainfluenzae* (*Olsen et al., 2005*). All strains were remote from the genus *Pasteurella* sensu stricto represented by the *P. multocida* type strain.

Sequence similarity of rat *Haemophilus* strains with Hayashimoto's strain V6 (accession number AB279579) and with [*P.*] *pneumotropica*-like MCCM 00235 (AF224300), amounted to 98-100% and 96-98% respectively.

Sequence similarity of rat and guinea pig *Haemophilus* strains with Bisgaards taxon 22 (accession number AY172726) appeared 98-99%.

Similarity of rat and guinea pig *Haemophilus* strains with *H. parainfluenzae* strain CIP 102513 (accession number EU083530) was 97 and 98% respectively for strains assigned to the two subclusters.

PCR using 3 primer sets

Bootz's (*Boot et al., 1998*) primer set detected all bacterial strains. The set aimed to detect Jawetz biotype of [*P.*] *pneumotropica* was positive with two rat strains resembling Hayashimoto's strain V6 (Fig. 1) The set for biotype Heyl [*P.*] *pneumotropica* did not detect any of the V-factor requiring strains. Bootz's primer set detected significantly more strains than both other sets (Fisher exact test, $p < 0.001$).

Phenotypic characteristics

The outcome of API NH profiling and cellular fatty acid analysis is given in Table 1 and Fig. 1. No rela-

tionship between phenotypic properties and assignment based on 16S rDNA sequences was apparent.

Comparison of PCR with culture and serology

Negative control rats from our hysterectomy-derived barrier-maintained breeding colony were negative for the last two years by culture, ELISA and PCR upon periodic monitoring according to the FELASA recommendations (Table 1).

From all other rat groups, V-factor requiring bacteria (*Haemophilus* spp) were cultured (Table 2), all rat groups contained ELISA positive animals, and all rats tested positive by PCR. The 3 test methods differed significantly in the detection of infected rats (Cochran's Q test, $p<0.001$; $n=76$). The *Pasteurellaceae* PCR appeared superior to both other test methods (McNemar test, $p<0.001$; $n=76$), but the number of infected rats detected by ELISA and culture was not different (McNemar test; $p=0.07$; $n=76$).

Considering the *Pasteurellaceae* PCR as the 'gold standard', the sensitivity of culture and serology were 64 and 80 % respectively (Table 2).

Negative control guinea pigs from our hysterectomy-derived barrier-maintained colony were negative for the last two years by culture, ELISA and PCR upon periodic monitoring according to the FELASA recommendations (Table 1).

From all other guinea pig groups examined V-factor dependent *Pasteurellaceae* (*Haemophilus* spp) were cultured (Table 3), all but one of the groups contained seropositive animals, and all guinea pigs tested positive by PCR. The 3 test methods differed significantly in the detection of infected guinea pigs (Cochran's Q test, $p<0.001$; $n=40$). The *Pasteurellaceae* PCR appeared superior to both other test methods (McNemar test, $p<0.001$; $n=40$), but the number of infected guinea pigs detected by ELISA and culture was not different (McNemar test; $p=0.1$; $n=40$). Considering the *Pasteurellaceae* PCR as the 'gold standard', the sensitivity of culture and serology were 64 and 80 % respectively (Table 2).

Discussion

Phylogenetic analysis

Haemophilus strain V6 (*Hayashimoto et al., 2007*), [*P.*] *pneumotropica*-like MCCM 00235 and Bisgaards taxon 22 class were within the Rodent cluster of the *Pasteurellaceae* (*Ryll et al., 1968*; *Olsen et al., 2005*). This implies that 8 out of 15 of our *Haemophilus* strains (5 from rat and 3 from guinea pig) could be assigned to the Rodent cluster.

H. parainfluenzae strain CIP 102513 is classed within the *H. parainfluenzae* complex so our remaining *Haemophilus* strains (4 from rat and 3 from guinea pig) could be assigned to this complex.

Our findings confirm earlier observations that V-factor requiring *Pasteurellaceae* from rodents may be inside (*Ryll et al., 1968, Hayashimoto et al., 2007*) and outside the Rodent cluster. Until now all rodent *Haemophilus* strains outside the Rodent cluster have been found related to *H. parainfluenzae* (*Nicklas et al., 1993; Hayashimoto et al., 2007*).

We studied only a limited number of bacterial strains by partial 16S rDNA sequencing. Rat and guinea pig *Haemophilus* strains comprise a large number of API NH/FAME phenotypes (*Boot, 2008*). It is therefore not surprising to find different *Haemophilus* phenotypes among strains assigned to each of the 16S rDNA based clusters (Fig. 1 and Table 1)

PCR detection by 3 primer sets

In our previous study Bootz's primer set detected all V-factor independent *Pasteurellaceae* strains studied (*Boot et al, 2009*). In the present study the primer set also detected all V-factor dependent bacterial strains (Table 1 or Fig. 1).

As in our previous study the PCRs using primer sets for Heyl biotype [*P.*] *pneumotropica* (*Kodjo et., 1998*) and for Jawetz biotype [*P.*] *pneumotrotropica* (*Wang et al., 1996*) appeared inferior.

Both groups of rodent *Pasteurellaceae*, namely growth factor dependent (this study) and independent strains (*Boot et al., 2009*), comprise strains that belong to clusters, which are widely dispersed over the *Pasteurellaceae* dendrogram (*Olsen et al., 2005*).

FELASA recommends the testing of rodents for all *Pasteurellaceae* taxa (*Nicklas et al., 2002).* If PCR is the method of choice obviously Bootz's primer set should be used.

Comparison of PCR with culture and serology

It is not surprising that all 3 methods were found suitable for the detection of *Haemophilus* infection in both species of animal (Tables 2 and 3).

That none of the rat and guinea pig colonies were reported infected by *Pasteurellaceae* is of concern as this may indicate that frequently *Haemophilus* infection remains undetected by animal suppliers. Another possibility is that (if detected) infection by *Haemophilus* is not reported, which would be in conflict with FELASA's recommendations for the health monitoring of rodent colonies (*Nicklas et al., 2002).*

In both species PCR appeared superior to culture and serology in detecting *Haemophilus* infected animals. *Bootz et al.* (1998) who did not use serology, found PCR superior to culture for detection of growth factor independent *Pasteurellaceae* infection. An opposite outcome (PCR is inferior) might be obtained using PCR primer sets that detect only a limited variety of *Pasteurellaceae*, for instance Kodjo's and Wangs set for Heyl and Jawetz biotype [*P.*] *pneumotropica* respectively.

Implications for monitoring

The percentage of positive tests (prevalence) found in rats and guinea pigs determines the likelihood of detecting an infected animal (*Nicklas et al., 2002*). Obviously the 100% prevalence found by *Pasteurellaceae* PCR implies that the examination of one animal only would have been sufficient to detect infection in a colony.

Traditionally cultural results have been considered the 'gold standard' to which the sensitivity of serology may be compared. The outcome of PCR testing of rodents for *Pasteurellaceae* infection using Bootz's primer set might be considered the new 'gold standard' to which the sensitivity of other testing can be compared.

References

Boot R, H Thuis & MA Koedam. Infection by V-factor dependent *Pasteurellaceae* (*Haemophilus*) in rats. J. Exp. Anim. Sci., 1994, *37*, 7-14.

Boot R, H Thuis, RHG Bakker & JL Veenema. An enzyme-linked immunosorbent assay (ELISA) for monitoring rodent colonies for *Pasteurella pneumotropica* antibodies. Lab. Anim. 1995, *29*, 307-313.

Boot R, H Thuis & JL Veenema. Serological relationship of some V-factor dependent *Pasteurellaceae* (*Haemophilus*) from guinea pigs and rabbits. Lab. Anim., 1998*, 33*, 91-94.

Boot R. Guinea pig and rat as carriers of host-unique and shared *Haemophilus* phenotypes. Scand. J. Lab. Anim. Sci., 2008, *35*, 163-167.

Boot R, MJ Vlemminx & FAG Reubsaet. Comparison of polymerase chain reaction primer sets for amplification of rodent *Pasteurellaceae*. Lab. Anim., 2009, *43*, 371-375.

Bootz F, S Kirschnek, W Nicklas, SK Wyss & FR Homberger. Detection of *Pasteurellaceae* in rodents by polymerase chain reaction analysis. Lab. Anim. Sci., 1998, *48*, 542-546.

Christensen H, G Foster, JP Christensen, T Pennycott, JE Olsen & M Bisgaard. Phylogenetic analysis by 16S rDNA gene sequence comparison of avian taxa of Bisgaard and characterization and description of two new taxa of *Pasteurellaceae*. J. Appl. Microbiol., 2003, *95*, 354-363.

Hayashimoto N, A Takakura & T Itoh. Genetic diversity on 16S rDNA sequence and phylogenetic tree analysis in *Pasteurella pneumotropica* strains isolated from laboratory animals. Current Microbiol., 2005, *51*, 239-243.

Hayashimoto N, M Ueno, A Takakura & T Itoh. Biochemical characterization and phylogenetic analysis based on 16S rRNA sequences for V-factor dependent members of *Pasteurellaceae* from laboratory rats. Current Microbiol., 2007, *54*, 419-423.

Kodjo A, L Villard, F Veillet, F Escande, E Borges, F Maurin, J Bonnod & Y Richard. Identification by 16S rDNA fragment amplification and deter-

mination of genetic diversity by random amplified polymorphic DNA analysis of *Pasteurella pneumotropica* isolated from laboratory rodents. Lab. Anim. Sci., 1998, *49*, 49-53.

Nicklas W. Haemophilus infection in a colony of laboratory rats. J. Clin. Microbiol., 1989, *27*, 1636-1639.

Nicklas W, M Staut & A Benner. Prevalence and biochemical properties of V factor dependent *Pasteurellaceae* from rodents. Zentralbl. Bakteriol., 1993, *279*, 114-124.

Nicklas W, P Baneux, R Boot, T Decelle, AA Deeny, B Illgen-Wilke & M Fumanelli. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab. Anim., 2002, *36*, 20-42.

Olsen I, FE Dewhirst, BJ Paster & HJ Busse. Pasteurellaceae Pp. 851-912 in: *Bergey's Manual of Systematic Bacteriology* vol. 2nd ed. Part B (Brenner DJ, Krieg NR, Staley JT eds). Springer, New York 2005.

Ryll M, R Muttters & W Mannheim. Untersuchungen zur genetischen Klassifikation des Pasteurella pneumotropica Komplexes. Bberl. Munch. Tierartzl. Wochenschr. 1968, *104*, 243-245.

Wang RF, W Campbell, WW Cao, C Summage, RS Steele & CE Cerniglia. Detection of *Pasteurella pneumotropica* in laboratory mice and rats by polymerase chain reaction. Lab. Anim. Sci. 1996, *46*, 81-85.