Development of a Quick and Easy Molecular Method for Differentiating *Haemophilus (Glässerella) parasuis* from Species Belonging to the Genus *Actinobacillus*

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ABSTRACT

A quick, easy, and economical molecular method, which is capable of differentiating *Haemophilus (Glässerella) parasuis* from several species belonging to genus *Actinobacillus*, is reported in this paper. These species are currently isolated from porcine airways, and show similar growth characteristics to those of *H. (G.) parasuis*. The proposed method involves a rapid extraction of RNA from the bacteria grown on agar plates followed by an agarose gel electrophoresis, where an unusual band pattern of the ribosomal RNA of *H. parasuis* can be seen. In this way, the 23S ribosomal RNA subunit is split into two bands, while the 23S ribosomal RNA subunit remained without splitting in the species belonging to the genus *Actinobacillus*.

INTRODUCTION

Haemophilus (Glässerella) parasuis is a gram-negative organism belonging to the family *Pasteurellaceae*. This bacterial species is a commensal found in the upper respiratory tract of swine. Under certain conditions, *H. (G.) parasuis* can evade host defenses and cause a septicemia known as Glässer's disease, which is characterized by meningitis, polyserositis, and polyarthritis.[1] However, *H. (G.) parasuis* can also act as a secondary pathogen causing pneumonia within porcine respiratory disease complex (PRDC).[2] Along with *H. parasuis*, among members of family *Pasteurellaceae* isolated from swine respiratory tract, there are both commensal species (*Actinobacillus minor, A. porcinus* or *A. indolicus*)[3] and potentially

Table 1. *Bacterial strains used in this work, indicating the presence or absence of the 23S rRNA subunit band.*

Bacterial strains	Band for 23S rRNA
H. parasuis No.4 (reference strain of serovar 1)	-
H. parasuis SW140 (reference strain of serovar 2)	-
H. parasuis SW114 (reference strain of serovar 3)	-
H. parasuis SW124 (reference strain of serovar 4)	-
H. parasuis Nagasaki (reference strain of serovar 5)	-
H. parasuis 131 (reference strain of serovar 6)	-
H. parasuis 174 (reference strain of serovar 7)	-
H. parasuis C5 (reference strain of serovar 8)	-
H. parasuis D74 (reference strain of serovar 9)	-
H. parasuis H555 (reference strain of serovar 10)	-
H. parasuis H465 (reference strain of serovar 11)	-
H. parasuis H425 (reference strain of serovar 12)	-
H. parasuis 84-17975 (reference strain of serovar 13)	-
H. parasuis 84-22113 (reference strain of serovar 14)	-
H. parasuis 84-15995 (reference strain of serovar 15)	-
A. pleuropneumoniae Shope 4074 (reference strain of serovar 1)	+
A. pleuropneumoniae 1536 (reference strain of serovar 2)	+
A. pleuropneumoniae 1421 (reference strain of serovar 3)	+
A. pleuropneumoniae M62 (reference strain of serovar 4)	+
A. pleuropneumoniae K17 (reference strain of serovar 5a)	+
A. pleuropneumoniae L20 (reference strain of serovar 5b)	+
A. pleuropneumoniae Femø (reference strain of serovar 6)	+
A. pleuropneumoniae WF83 (reference strain of serovar 7)	+
A. pleuropneumoniae 405 (reference strain of serovar 8)	+
A. pleuropneumoniae CVJ13261 (reference strain of serovar 9)	+
A. pleuropneumoniae D13039 (reference strain of serovar 10)	+
A. pleuropneumoniae 56163 (reference strain of serovar 11)	+
A. pleuropneumoniae 8329 (reference strain of serovar 12)	+
A. indolicus 46KC2	+
A. minor H5 487	+
A. porcinus H223	+
A. suis 3063	+
A. seminis NTCC10851	+
A. ligneriesii (field strain)	+
A. rossii (field strain)	+
A. equuli (field strain)	+
A. ureae (field strain)	+
P. multocida (field strain, type A)	+
P. multocida (field strain, type D)	+
Trueperella pyogenes 2194/02 (field strain)	+
Streptococcus suis 1440/02	+
Bordetella bronchiseptica 157/9	+
Klebsiella pneumoniae ATCC13883	+

pathogenic species that may be also involved in the PRDC (such as *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*),[4] or in systemic infections with serositis and arthritis (*A. suis*).[5] Many species from genus *Actinobacillus* show colony morphologies that are very similar to those of *H. (G.) parasuis*. This often makes the identification of this latter species hard.[6]

The procedure used formerly to differentiate *H. (G.) parasuis* from the organisms belonging to genus *Actinobacillus* was based on the absence of hemolysis, negative CAMP test, and biochemical tests such as negative urease an indole, and positive catalase, resulting in a long and tedious method.[7] Subsequently, polymerase chain reaction (PCR) has allowed the differentiation between *H. (G.) parasuis* and bacteria from genus *Actinobacillus*.[8-12]

In this study, a rapid, simple and inexpensive methodology is described, intended to differentiate *H. (G.) parasuis* from other bacteria isolated from porcine airways, including particularly those of genus *Actinobacillus*. It is based on the split of 23S ribosomal RNA (rRNA) into two typical parts of certain bacterial species from genus *Haemophilus*.[13,14]

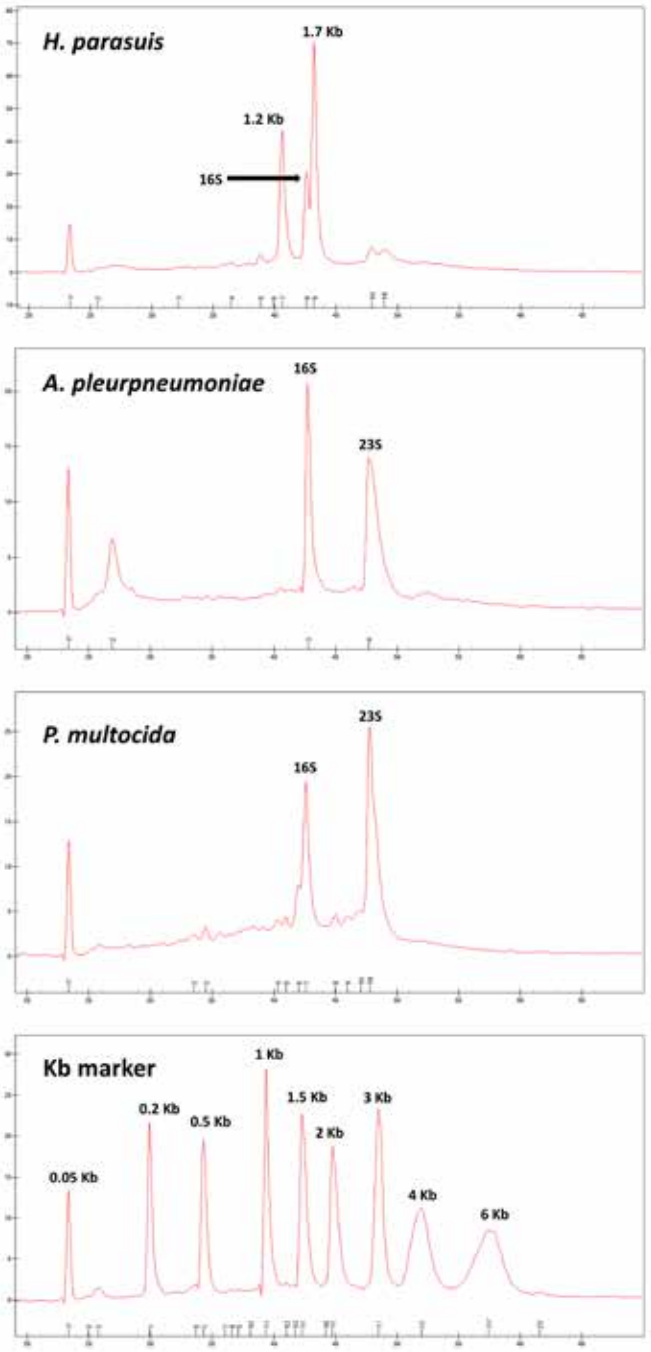
MATERIAL AND METHODS

H. (G.) parasuis Nagasaki strain, *A. pleuropneumoniae* 405 strain, and *P. multocida* NCTC 10322 strain were grown at 37˚C for 24 hours in 30 ml of PPLO broth (Laboratorios Conda, Spain), supplemented with 150 µM nicotinamide adenine dinucleotide (Sigma-Aldrich, Merck, Germany) and 0.075% glucose (Sigma-Aldrich). The extraction of RNA from these growths was performed using the High Pure RNA Isolation kit (Roche, Spain), in accordance with the manufacturer's specifications. Then, an automated electrophoresis was performed in order to characterize the band patterns of rRNA from these species. This procedure was carried out at the Laboratory for Instrumental Techniques of the University of Leon, Spain, using a Bioanalyzer Agilent 2100 (Agilent, CA).

Thereafter, on the basis of a previously described method for extracting RNA,[15] a faster and simpler protocol was developed and adapted for RNA extraction from growth on plates. The sole objective of the extraction of RNA with this method was the displaying of rRNA bands in agarose gel electrophoresis. Thus, the steps intended to improve the quality and purity of RNA could be avoided, resulting in a quicker and easier process. The protocol was the following: a sample of growth was taken with a loop and suspended in 200 µl of a buffer consisting of 0.1% N-lauryl sarcosine sodium salt (Sigma-Aldrich) in H2O, and collected by centrifugation at 10,000× g for 5 minutes at room temperature (RT). The pellet was then suspended in 100 µl of the second buffer, composed of 1% SDS (Sigma-Aldrich), 10 mM EDTA (Sigma-Aldrich), and 50 mM sodium acetate (Sigma-Aldrich), with a pH 5.1 adjusted with glacial acetic acid (Sigma-Aldrich). The suspension was boiled at 100°C for 5 minutes, and then was diluted with 650 µl of H2O and centrifuged at 7,000× g for 5 minutes at RT. A volume of 500 µl of supernatant containing RNA was collected and used for the subsequent agarose gel electrophoresis aimed at displaying the bands of rRNA.

Prior to electrophoresis, RNA concentrations were measured using a NanoDrop 1000 (Thermo Fisher Scientific, MA). The gels were prepared using TBE and 1.5% agarose, and were dyed with Red Safe (Intron Biotechnology, Korea). To perform the electrophoresis, 27 µl of sample, with a minimum of 2 µg of RNA, was mixed with 3 µl of 10x loading buffer (Takara Bio Inc, Japan) and heated at 70ºC for 10 minutes to denature the RNA. The full volume of denatured sample was then loaded into the gel and electrophoresis was carried out at 100 v for 45 minutes. This new proposed methodology was applied to the 43 bacterial strains used in this study that are shown in Table 1. All of them were grown on chocolate agar plates (Oxoid, Thermo Fisher Scientific, Waltham, MA) for 24 hours at 37˚C and 5% CO2.

Figure 1. *Automated electrophoresis showing peaks of rRNA from H. parasuis, A. pleuropneumoniae and P. multocida. Last panel shows the molecular-weight size marker. Y axis corresponds to fluorescence, X axis corresponds to time (in seconds). A. pleuropneumoniae and P. multocida presented peaks corresponding to the prokaryotic 16S (1.5 kb) and 23S (2.9 kb) rRNA subunits. H. parasuis showed the 16S peak (1.5 kb) but did not the 23S rRNA peak. However, two peaks about 1.2 kb and 1.7 kb were observed.*



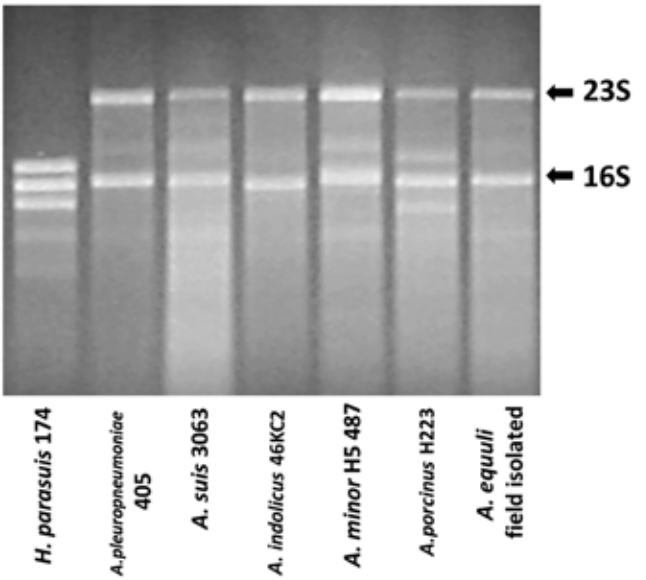
RESULTS AND DISCUSSION

The results of the proposed electrophoresis showed that *A. pleuropneumoniae* and *P. multocida* presented peaks corresponding to the prokaryotic 16S and 23S rRNA subunits (about 1.5 kb and 2.9 kb, respectively). In contrast, *H. (G.) parasuis* showed the 16S peak but did not show the 23S peak. Instead of this, two peaks about 1.2 kb and 1.7 kb were observed (Fig. 1).

The results of agarose gel electrophoresis showed that all the reference strains of the 15 serovars of *H. (G.) parasuis* exhibited the band corresponding to 16S rRNA subunit, but did not show the band corresponding to 23S rRNA subunit (only the reference strain of serovar 7 of *H. (G.) parasuis* is presented in Fig. 2). They showed in place two bands derived from a split in 23 rRNA subunit.

In contrast, all the strains belonging to the genus *Actinobacillus* tested, including the species isolated from various other types of mammals, showed a band corresponding to the 23S rRNA subunit (Fig. 2). This fact demonstrates that its integrity is conserved through genus *Actinobacillus*. In addition, *A. ureae*, *A. minor*, *A. rossii*

Figure 2. *Agarose gel electrophoresis. The first lane corresponds to the rRNA bands from H. parasuis 174 strain and shows the disappearance of the 23S band. This was found in all 15 H. parasuis strains tested. The second lane corresponds to the rRNA bands from A. pleuropneumoniae 405 (reference strain of serovar 7) and shows a 23S band. This was also found in all 14 other A. pleuropneumoniae strains tested. The third to seventh lanes corresponded to the rRNA bands from various species belonging to genus Actinobacillus and they showed a 23S band. The remaining species tested were not included in the image, but also showed a 23S rRNA band.*



and *A. porcinus* also displayed additional bands, which might be due to intra-genomic variability among multiple rRNA genes encoded on a single genome, resulting in both intact and fragmented rRNA subunits. Nevertheless, this finding did not affect the purpose of this study because all of these species showed a band corresponding to the 23S rRNA subunit, regardless of exhibiting additional bands or not. Other species commonly isolated from the porcine respiratory tract, such as *P. multocida* (types A and D), *Trueperella pyogenes*, *Streptococcus suis*, *Klebsiella pneumoniae*, and *Bordetella bronchiseptica*, were also included in this study, and showed an intact 23S rRNA band (Table 1).

The method here described is fast and simple, because the time required to perform it is less than 2 hours, including bacterial growth from plates, sampling of RNA extraction, agarose gel electrophoresis, and band pattern visualization. Furthermore, it is an inexpensive method because all of the reagents and equipment required are commonly found in microbiological laboratories. Therefore, this method may constitute an easy alternative to PCR-based methods or laborious biochemical tests in differentiating *H. (G.) parasuis* from species belonging to genus *Actinobacillus*. Most of them are also often isolated from the porcine respiratory tract, especially A. pleuropneumoniae, a major porcine respiratory pathogen included as primary pathogen in the PRDC.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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