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Specific identification of *Gallibacterium* by a PCR using primers targeting the 16S rRNA and 23S rRNA genes

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Running title: Identification of *Gallibacterium* by PCR.
Abstract

Gallibacterium was recently established as a new genus including organisms previously reported as Pasteurella anatis, [Actinobacillus] salpingitidis and avian Pasteurella haemolytica-like organisms. The aim of the present study was to develop a PCR method allowing unambiguous identification of Gallibacterium. PCR primers positioned in the 16S rRNA (1133fgal) and 23S rRNA (114r) genes were defined and their specificity was subsequently tested on 122 strains. Twenty-five of the strains represented all of the presently available 15 phenotypic variants of Gallibacterium from different geographical locations, 22 other strains represented other poultry associated bacterial species or bacteria which could pose a differential diagnostic problem including members of the families Pasteurellaceae, Enterobacteriaceae and Flavobacteriaceae, and finally 75 Gallibacterium field strains isolated from Mexican chicken egg-layers. Specific amplicons were generated in all 100 Gallibacterium strains tested, whereas none of the non-Gallibacterium strains tested positive. Correct identification was confirmed by hybridization with the Gallibacterium specific probe GAN850.

Two internal amplification control strategies were successfully incorporated into the PCR assay, one based on amplification of the house-keeping gene rpoB (sharing target DNA) and another based on addition of trout DNA (foreign target DNA) and amplification with β-actin specific primers.

In conclusion, the described PCR assay enables specific identification of Gallibacterium and will thus stand as a strong alternative to the present diagnostic methods.

Keywords: Gallibacterium; PCR identification; ITS-PCR
1. Introduction

Bacteria previously classified as [Actinobacillus] salpingitidis, Pasteurella haemolytica-like or Pasteurella anatis have recently been reclassified and relocated into a new genus, Gallibacterium, of the family Pasteurellaceae Pohl 1981 (Christensen et al., 2003). Presently, the genus includes the species, Gallibacterium anatis and Gallibacterium genomospecies 1 and 2.

Gallibacterium anatis has a haemolytic and a non-haemolytic form termed biovars haemolytica and anatis, respectively. Gallibacterium anatis biovar haemolytica has been isolated from healthy birds (Harry, 1962; Bisgaard, 1977; Mushin et al., 1980; Bojesen et al., 2003a) and although the pathogenic potential of G. anatis biovar anatis is currently not fully understood, a number of isolates have been obtained from diseased birds and cattle (Bisgaard, 1982; Bisgaard, 1993; Lin et al., 2001; Christensen et al., 2003). In particular, these organisms seem to be implicated in salpingitis, peritonitis and oophoritis in poultry (Mirle et al., 1991; Jordan et al., 2005; Vazquez et al., 2006). Identification of members of Pasteurellaceae by traditional means are characterized by difficulties at isolation, culturing and weak reactions toward some of the phenotypic tests used for identification (Christensen et al., 2003).

Identification of Gallibacterium is at present best performed through the phenotypic characterising outlined in Christensen et al. (2003) or by the use of the Gallibacterium specific probe, GAN850 (Bojesen et al., 2003b). In addition, it is difficult to separate the non-haemolytic isolates from Avibacterium gallinarum, whereas separation of haemolytic isolates from other taxa is less problematic. Gallibacterium includes a few bovine isolates, which previously have been misclassified as Pasteurella multocida since ornithine decarboxylase and indole negative isolates of P. multocida subsp. septica have the same phenotype as G. anatis.

Consequently, the aim of the present study was to develop a PCR assay specific for Gallibacterium allowing rapid and unambiguous identification. Gallibacterium has a relatively short internal transcribed 16S to 23S rRNA gene sequence compared to other members of Pasteurellaceae and this was used in the current investigation (Gurtler and Stanisich, 1996; Christensen et al., 2003). We used the primers 114r located on the 23S rRNA gene and
1133fgal located on the 16S rRNA gene to demonstrate specific amplification of one or two
fragments corresponding to one or two ribosomal operon sizes in *Gallibacterium*. The results
from the PCR were compared with results from hybridization with the *Gallibacterium* specific
probe (Bojesen *et al.*, 2003b) and we found 100% agreement between the two identification
methods.

In conclusion, we demonstrate an identification method based on primers specifically targeting
the 16S and 23S rRNA genes in *Gallibacterium*. The specificity of the method was confirmed
by negative PCR’s with 22 other poultry associated bacterial species or related members of the
families *Pasteurellaceae*, *Enterobacteriaceae* and *Flavobacteriaceae*.

2. Material and methods

2.1. Bacterial strains

A total of 122 strains including 47 reference strains and 75 field isolates were investigated. The
reference strains included 25 *Gallibacterium* strains representing the broadest phenotypic and
genotypic diversity known within the genus. In addition, 17 related strains representing the
family *Pasteurellaceae* and five strains belonging to the families *Flavobacteriaceae* and
*Enterobacteriaceae*, which could represent a differential diagnostic problem, were included
(Table 1). The field strains originated from a total of 18 egg-laying flocks from different
Mexican states wherefrom diagnostic material had been submitted to the Boehringer Ingelheim
Laboratory in Guadalajara during 2000-2006. All flocks had experienced lowered egg
production.

2.2. Genus specific primers

Oligonucleotide primers were designed, based on ninety-nine 16S rRNA sequences from
GenBank (Benson *et al.*, 2004), representing all *Gallibacterium* and related members of
*Pasteurellaceae*. The primer 1133fgal (5’-TATTCTTTGTTACCARCGG) was predicted to be
specific for *Gallibacterium* and not able to amplify DNA of other members of *Pasteurellaceae*
accepted. For the reverse amplification, the general 23S rRNA gene sequence primer 114r (5'-GGTTTCCCCATTCGG) was chosen (Lane, 1991). The positions of the 16S rRNA gene sequence refer to the *Escherichia coli* *rrnB*. Comparison with the published ITS fragment length of *Gallibacterium* of 258, 454 and 501 bp gives predicted amplicons of 789, 985 and 1032 bp, respectively, by including the overhangs with 16S rRNA and 23S rRNA gene sequences.

2.3. **Internal Amplification Control (IAC)**

Two IAC were tested in the assay. One based on the house-keeping gene *rpoB*, which is widely distributed in Gram-negative genera, including *Gallibacterium*. The conserved primers 5'-GCAGTGAAGARTTCTTTGGTTC and 5'-GTTGCATGTTIGIACCCAT were used to amplify a product of 560 bp, according to Korzak *et al.* (2004). The second IAC tested was based on adding completely unrelated DNA from rainbow trout (*Oncorhyncus mykiss*) and the primers 5'-ATGGAAGATGAAATCGCC and 5'-TGCCAGATCTTCTCCATG targeting the highly conserved β-actin gene were added to the reaction mixture generating an amplicon of approx. 570 bp (Lindenstrøm *et al.*, 2003).

2.4. **Extraction of DNA and PCR conditions**

Bacteria were stored at –80 °C and cultivated overnight on blood agar base (Oxoid, Hampshire, UK) with 5% citrated bovine blood. Single colonies were cultured in Brain Heart Infusion broth (Oxoid) with shaking at 37 °C. The chelex extraction procedure of Widjojoatmodjo *et al.* (1994) was followed. Briefly, a bacterial colony from a blood-plate was added to 0.7 ml 10 % Chelex-100 solution. The solution was mixed after addition of 0.1 ml of 0.3 % SDS, 0.1 ml of 10% Tween 20 and 0.1 ml of 10% Nonidet P-40. The solution was boiled for 5 min and centrifuged. Two microlitres of supernatant was used per PCR reaction. The Chelex lysates were stored at 4 °C and boiling was repeated prior to their use.
A Gene amp 9700 PCR machine (Applied Biosystems) was used. The PCR was set up with final concentrations of 1.5 mM MgCl$_2$, 1 X PCR reaction buffer, 200 µM dNTP, 1.5 U Taq enzyme and 0.5 µM of each oligonucleotide primers per reaction in a total reaction volume of 50 µl. The samples were denatured at 95°C for 4 min and the PCR was run for 35 cycles with 95°C denaturation for 30 s, 55°C annealing for 1 min and 72°C extension for 2 min. The PCR was terminated at 72°C extension for 10 min. The effect of different annealing temperatures were tested in the range of 55.0 to 60.1 °C at the specific temperatures of 55.0, 55.9, 56.8, 57.8, 58.8 and 60.1 °C on a temperature gradient Thermo Hybaid PCR Express machine. The PCR products were analysed on a 1% agarose gel and stained with ethidium bromide.

2.5. Hybridization of bacterial cells with a Gallibacterium specific probe
The Gallibacterium isolates were hybridized with the probe GAN850 specifically binding to the 16S rRNA of Gallibacterium and the bacterial probe EUB338 in accordance with the protocol previously described in Bojesen et al. (2003b).

3. Results
The primer set 1133fgal – 114r was specific for the strains of Gallibacterium tested under the PCR conditions specified in the Material and Methods and did not generate PCR products in any of non-Gallibacterium strains (Fig. 1). A PCR amplicon of approx. 790 bp was present in all Gallibacterium isolates tested. A second amplicon of approx. 1080 bp was present in all 25 reference strains but missing in 14 out of 75 of the Mexican field strains. The size of the larger amplicon was variable, ranging from 1030 bp to 1080 bp, with the larger amplicon present in G. anatis and the smaller in G. genomospecies 1 and 2 (Fig. 1). Changing the annealing temperature between 55.0 and 60.1 °C did not alter the overall performance of the assay (Data not shown). The IAC based on the rpoB gene generated an amplicon of approx. 560 bp (Fig. 1A), whereas the IAC based on the β-actin gene in trout (Oncorhyncus mykiss) generated an amplicon of approx. 570 bp (Fig. 1B). There was 100% agreement between the results obtained
by hybridization with the *Gallibacterium* specific probe, GAN850, and the PCR assay, resulting in the PCR method being 100% specific and 100% sensitive in the present investigation.

4. Discussion

Genus *Gallibacterium* is a very diverse group of bacteria comprising isolates which vary in phenotypical characteristics, the hosts from which they are isolated, geographical location and time of isolation. The present strain collection, to the authors’ knowledge, is the most diverse and complete to date. However, this does not guarantee that the present PCR assay will perform without fail in all instances, as mutations can alter the priming sites or isolates in which sequences differ slightly. Inclusion of negative controls are therefore very important, however, inclusion of a positive control, like an IAC, is also very useful under non-culturable conditions (Malorny and Hoorfar, 2005). In the present investigation, an IAC based on identical target DNA and another based on foreign target DNA was tested (Hoorfar et al., 2004). Both protocols generated the expected amplicons under the conditions tested and therefore seem suitable. In the case of the *rpoB* based IAC, the specific primers were designed to fit to the *Pasteurellaceae rpoB* gene sequences and although this gene is highly conserved, sequence diversity in distantly related bacteria may cause inability of the primers to anneal to the *rpoB* genes and thus giving a false negative IAC result. This problem should not be expected using the alternative IAC tested in the present study as the target, i.e. trout DNA which was added to every reaction mixture. Using trout DNA there is little risk of getting a false positive reactions from contamination by i.e. host derived DNA.

The present ITS-PCR generally produced two products with sizes of approx. 790 bp and 1080 bp in the *Gallibacterium* strains tested. However, in 14 out of 75 field strains, only one fragment (approx. 790 bp) appeared. In addition, a third intermediate size fragment has also been reported from a single bovine isolate (Hom. 33) (Christensen et al., 2003), however, none of the strains in the present investigation, including two of bovine origin, seemed to possess a third size ribosomal operon. The variability in the ITS number could be due to genuine
differences in the sizes of the ITS's, but could also be due to sequence differences among the
different ribosomal operons present not allowing the primers to bind to more than one or two
operon types.

The *Gallibacterium* specific ITS-PCR not only selectively amplifies *Gallibacterium* DNA it
also generates relatively short fragments when compared to other members of *Pasteurellaceae*
(Leys et al., 1994; Fussing et al., 1998; Gu et al., 1998; Christensen et al., 2003). ITS
fragments shorter than 350 bp have so far only been recorded in two bovine isolates of
*Pasteurella multocida* (Strains 66 and 248) (Christensen et al., 2004), making the size in itself a
good identifier. The amplicon sizes of 790 bp and 1080 bp subtracted the overlap from the
priming sites in the 16S and the 23S rRNA genes, corresponded well with the sizes of approx.
250 bp and of approx. 450 to 500 bp, previously reported by Christensen et al. (2003). The size
variation of the larger amplicon seemed to some extent to be species specific, meaning that *G.
anatis* strains possessed the larger amplicon (approx. 1080 bp) whereas *G. genomospecies 1
and 2* possessed the smaller (1030 bp). Whether this result can be used as a general rule
enabling species determination based the on the size of the larger amplicon remains uncertain
until further strains representing *G. genomospecies 1 and 2* have been made available.

In conclusion, the present PCR assay for specific identification of *Gallibacterium* was tested on
a very diverse collection of *Gallibacterium* strains, all of which tested positive. More
importantly, all the included related organisms which could represent a differential diagnostic
problem tested negative. The assay therefore seems very useful for unambiguous identification
of members of genus *Gallibacterium* in routine diagnostics.

5. Acknowledgements

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Christensen, H., Angen, Ø., Olsen, J. E., Bisgaard, M. 2004. Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. Microbiology. 150, 1757-1767.


Gurtler, V. and Stanisich. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology. 142, 3-16.


Figure legends

Fig. 1.

A) PCR amplicons from *Gallibacterium anatis* biovar *anatis* (F149T), *G. anatis* biovar *haemolytica* (12656-12), *G.* genomospecies 1 (CCM5974), *G.* genomospecies 2 (CCM5976) and *Pasteurella multocida* subsp. *multocida* (NCTC10322T). The amplicon of 790 bp is present in all *Gallibacterium* strains tested. The larger amplicon in *G. anatis* (approx. 1080 bp) was slightly smaller in *G.* genomospecies 1 and *G.* genomospecies 2 (approx. 1030 bp). The internal amplification control based on the *rpoB* gene generated an amplicon of approx. 520 bp.

B) PCR amplicons from *Gallibacterium anatis* biovar *anatis* (F149T), *G. anatis* biovar *haemolytica* (12656-12), *G.* genomospecies 1 (CCM5975), *G.* genomospecies 2 (CCM5976) and *Pasteurella multocida* subsp. *multocida* (NCTC10322T). The internal amplification control based on amplification of the β-actin gene in trout (*Oncorhyncus mykiss*) DNA generated an amplicon of approx. 570 bp. The O’Generuler 1 Kb DNA ladder was used as size marker.
Table 1. Bacterial reference strains included in this investigation.

<table>
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<td>Pneumonia</td>
<td>Sparrow Hawk</td>
<td>DK</td>
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<td>B301529/00/1</td>
<td>Bisgaard Taxon 40</td>
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<td>Respiratory tract</td>
<td>Seagull</td>
<td>Scotland</td>
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<td>4237/2sv</td>
<td>Riemereella anatipesfer</td>
<td>Pharynx</td>
<td>Duckling</td>
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<td>G9</td>
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<td>Septicaemia</td>
<td>Chicken</td>
<td>UK</td>
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<td>Escherichia coli</td>
<td>Intestine</td>
<td>Human</td>
<td>Unknown</td>
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<tr>
<td>726-82&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Coenonia anatina</td>
<td>Unknown</td>
<td>Duck</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>CUG23171&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Ornithobacterium rhinoracheale</td>
<td>Respiratory tract</td>
<td>Turkey</td>
<td>UK</td>
<td></td>
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<sup>a</sup>B: Belgium, Can: Canada, Cz: Czech republic, DK: Denmark, G: Germany, UK: United kingdom. <sup>T</sup> Type strain
Figure 1