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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.

1 **Abstract**

2 *Gallibacterium* was recently established as a new genus including organisms previously
3 reported as *Pasteurella anatis*, [*Actinobacillus*] *salpingitidis* and avian *Pasteurella*
4 *haemolytica*-like organisms. The aim of the present study was to develop a PCR method
5 allowing unambiguous identification of *Gallibacterium*. PCR primers positioned in the 16S
6 rRNA (1133fgal) and 23S rRNA (114r) genes were defined and their specificity was
7 subsequently tested on 122 strains. Twenty-five of the strains represented all of the presently
8 available 15 phenotypic variants of *Gallibacterium* from different geographical locations, 22
9 other strains represented other poultry associated bacterial species or bacteria which could pose
10 a differential diagnostic problem including members of the families *Pasteurellaceae*,
11 *Enterobacteriaceae* and *Flavobacteriaceae*, and finally 75 *Gallibacterium* field strains isolated
12 from Mexican chicken egg-layers. Specific amplicons were generated in all 100 *Gallibacterium*
13 strains tested, whereas none of the non-*Gallibacterium* strains tested positive. Correct
14 identification was confirmed by hybridization with the *Gallibacterium* specific probe GAN850.
15 Two internal amplification control strategies were successfully incorporated into the PCR
16 assay, one based on amplification of the house-keeping gene *rpoB* (sharing target DNA) and
17 another based on addition of trout DNA (foreign target DNA) and amplification with β -actin
18 specific primers.

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

21

22 **Keywords:** *Gallibacterium*; PCR identification; ITS-PCR

1 **1. Introduction**

2 Bacteria previously classified as [*Actinobacillus*] *salpingitidis*, *Pasteurella haemolytica*-like or
3 *Pasteurella anatis* have recently been reclassified and relocated into a new genus,
4 *Gallibacterium*, of the family *Pasteurellaceae* Pohl 1981 (Christensen *et al.*, 2003). Presently,
5 the genus includes the species, *Gallibacterium anatis* and *Gallibacterium* genomospecies 1 and
6 2. *Gallibacterium anatis* has a haemolytic and a non-haemolytic form termed biovars
7 *haemolytica* and *anatis*, respectively. *Gallibacterium anatis* biovar *haemolytica* has been
8 isolated from healthy birds (Harry, 1962; Bisgaard, 1977; Mushin *et al.*, 1980; Bojesen *et al.*,
9 2003a) and although the pathogenic potential of *G. anatis* biovar *anatis* is currently not fully
10 understood, a number of isolates have been obtained from diseased birds and cattle (Bisgaard,
11 1982; Bisgaard, 1993; Lin *et al.*, 2001; Christensen *et al.*, 2003). In particular, these organisms
12 seem to be implicated in salpingitis, peritonitis and oophoritis in poultry (Mirle *et al.*, 1991;
13 Jordan *et al.*, 2005; Vazquez *et al.*, 2006). Identification of members of *Pasteurellaceae* by
14 traditional means are characterized by difficulties at isolation, culturing and weak reactions
15 toward some of the phenotypic tests used for identification (Christensen *et al.*, 2003).
16 Identification of *Gallibacterium* is at present best performed through the phenotypic
17 characterising outlined in Christensen *et al.* (2003) or by the use of the *Gallibacterium* specific
18 probe, GAN850 (Bojesen *et al.*, 2003b). In addition, it is difficult to separate the non-
19 haemolytic isolates from *Avibacterium gallinarum*, whereas separation of haemolytic isolates
20 from other taxa is less problematic. *Gallibacterium* includes a few bovine isolates, which
21 previously have been misclassified as *Pasteurella multocida* since ornithine decarboxylase and
22 indole negative isolates of *P. multocida* subsp. *septica* have the same phenotype as *G. anatis*.
23 Consequently, the aim of the present study was to develop a PCR assay specific for
24 *Gallibacterium* allowing rapid and unambiguous identification. *Gallibacterium* has a relatively
25 short internal transcribed 16S to 23S rRNA gene sequence compared to other members of
26 *Pasteurellaceae* and this was used in the current investigation (Gurtler and Stanisich, 1996;
27 Christensen *et al.*, 2003). We used the primers 114r located on the 23S rRNA gene and

1 1133fgal located on the 16S rRNA gene to demonstrate specific amplification of one or two
2 fragments corresponding to one or two ribosomal operon sizes in *Gallibacterium*. The results
3 from the PCR were compared with results from hybridization with the *Gallibacterium* specific
4 probe (Bojesen *et al.*, 2003b) and we found 100% agreement between the two identification
5 methods.

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

10

11 **2. Material and methods**

12 2.1. *Bacterial strains*

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

22

23 2.2. *Genus specific primers*

24 Oligonucleotide primers were designed, based on ninety-nine 16S rRNA sequences from
25 GenBank (Benson *et al.*, 2004), representing all *Gallibacterium* and related members of
26 *Pasteurellaceae*. The primer 1133fgal (5'-TATTCTTTGTTACCARCGG) was predicted to be
27 specific for *Gallibacterium* and not able to amplify DNA of other members of *Pasteurellaceae*

1 under the PCR conditions chosen. For the reverse amplification, the general 23S rRNA gene
2 sequence primer 114r (5'-GGTTTCCCCATTCGG) was chosen (Lane, 1991). The positions of
3 the 16S rRNA gene sequence refer to the *Escherichia coli rrmB*. Comparison with the published
4 ITS fragment length of *Gallibacterium* of 258, 454 and 501 bp gives predicted amplicons of
5 789, 985 and 1032 bp, respectively, by including the overhangs with 16S rRNA and 23S rRNA
6 gene sequences.

7

8 2.3. Internal Amplification Control (IAC)

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

17

18 2.4. Extraction of DNA and PCR conditions

19 Bacteria were stored at -80 °C and cultivated overnight on blood agar base (Oxoid, Hampshire,
20 UK) with 5% citrated bovine blood. Single colonies were cultured in Brain Heart Infusion broth
21 (Oxoid) with shaking at 37 °C. The chelex extraction procedure of Widjojoatmodjo *et al.* (1994)
22 was followed. Briefly, a bacterial colony from a blood-plate was added to 0.7 ml 10 % Chelex-
23 100 solution. The solution was mixed after addition of 0.1 ml of 0.3 % SDS, 0.1 ml of 10%
24 Tween 20 and 0.1 ml of 10% Nonidet P-40. The solution was boiled for 5 min and centrifuged.
25 Two microlitres of supernatant was used per PCR reaction. The Chelex lysates were stored at 4
26 °C and boiling was repeated prior to their use.

1 A Gene amp 9700 PCR machine (Applied Biosystems) was used. The PCR was set up with
2 final concentrations of 1.5 mM MgCl₂, 1 X PCR reaction buffer, 200 μM dNTP, 1.5 U Taq
3 enzyme and 0.5 μM of each oligonucleotide primers per reaction in a total reaction volume of
4 50 μl. The samples were denatured at 95°C for 4 min and the PCR was run for 35 cycles with
5 95°C denaturation for 30 s, 55°C annealing for 1 min and 72°C extension for 2 min. The PCR
6 was terminated at 72°C extension for 10 min. The effect of different annealing temperatures
7 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,
8 58.8 and 60.1 °C on a temperature gradient Thermo Hybaid PCR Express machine. The PCR
9 products were analysed on a 1% agarose gel and stained with ethidium bromide.

10

11 2.5. Hybridization of bacterial cells with a *Gallibacterium* specific probe

12 The *Gallibacterium* isolates were hybridized with the probe GAN850 specifically binding to
13 the 16S rRNA of *Gallibacterium* and the bacterial probe EUB338 in accordance with the
14 protocol previously described in Bojesen *et al.* (2003b).

15

16 3. Results

17 The primer set 1133fgal – 114r was specific for the strains of *Gallibacterium* tested under the
18 PCR conditions specified in the Material and Methods and did not generate PCR products in
19 any of non-*Gallibacterium* strains (Fig. 1). A PCR amplicon of approx. 790 bp was present in
20 all *Gallibacterium* isolates tested. A second amplicon of approx. 1080 bp was present in all 25
21 reference strains but missing in 14 out of 75 of the Mexican field strains. The size of the larger
22 amplicon was variable, ranging from 1030 bp to 1080 bp, with the larger amplicon present in *G.*
23 *anatis* and the smaller in *G.* genomospecies 1 and 2 (Fig. 1). Changing the annealing
24 temperature between 55.0 and 60.1 °C did not alter the overall performance of the assay (Data
25 not shown). The IAC based on the *rpoB* gene generated an amplicon of approx. 560 bp (Fig.
26 1A), whereas the IAC based on the β -actin gene in trout (*Oncorhynchus mykiss*) generated an
27 amplicon of approx. 570 bp (Fig. 1B). There was 100% agreement between the results obtained

1 by hybridization with the *Gallibacterium* specific probe, GAN850, and the PCR assay, resulting
2 in the PCR method being 100% specific and 100% sensitive in the present investigation.

3

4 **4. Discussion**

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

22 The present ITS-PCR generally produced two products with sizes of approx. 790 bp and 1080
23 bp in the *Gallibacterium* strains tested. However, in 14 out of 75 field strains, only one
24 fragment (approx. 790 bp) appeared. In addition, a third intermediate size fragment has also
25 been reported from a single bovine isolate (Hom. 33) (Christensen *et al.*, 2003), however, none
26 of the strains in the present investigation, including two of bovine origin, seemed to possess a
27 third size ribosomal operon. The variability in the ITS number could be due to genuine

1 differences in the sizes of the ITS's, but could also be due to sequence differences among the
2 different ribosomal operons present not allowing the primers to bind to more than one or two
3 operon types.

4 The *Gallibacterium* specific ITS-PCR not only selectively amplifies *Gallibacterium* DNA it
5 also generates relatively short fragments when compared to other members of *Pasteurellaceae*
6 (Leys *et al.*, 1994; Fussing *et al.*, 1998; Gu *et al.*, 1998; Christensen *et al.*, 2003). ITS
7 fragments shorter than 350 bp have so far only been recorded in two bovine isolates of
8 *Pasteurella multocida* (Strains 66 and 248) (Christensen *et al.*, 2004), making the size in itself a
9 good identifier. The amplicon sizes of 790 bp and 1080 bp subtracted the overlap from the
10 priming sites in the 16S and the 23S rRNA genes, corresponded well with the sizes of approx.
11 250 bp and of approx. 450 to 500 bp, previously reported by Christensen *et al.* (2003). The size
12 variation of the larger amplicon seemed to some extent to be species specific, meaning that *G.*
13 *anatis* strains possessed the larger amplicon (approx. 1080 bp) whereas *G.* genomospecies 1
14 and 2 possessed the smaller (1030 bp). Whether this result can be used as a general rule
15 enabling species determination based the on the size of the larger amplicon remains uncertain
16 until further strains representing *G.* genomospecies 1 and 2 have been made available.
17 In conclusion, the present PCR assay for specific identification of *Gallibacterium* was tested on
18 a very diverse collection of *Gallibacterium* strains, all of which tested positive. More
19 importantly, all the included related organisms which could represent a differential diagnostic
20 problem tested negative. The assay therefore seems very useful for unambiguous identification
21 of members of genus *Gallibacterium* in routine diagnostics.

22

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11

12

1 **Figure legends**

2

3 **Fig. 1.**

4 **A)** PCR amplicons from *Gallibacterium anatis* biovar *anatis* (F149^T), *G. anatis* biovar
5 *haemolytica* (12656-12), *G. genomospecies 1* (CCM5974), *G. genomospecies 2*
6 (CCM5976) and *Pasteurella multocida* subsp. *multocida* (NCTC10322^T). The amplicon of
7 790 bp is present in all *Gallibacterium* strains tested. The larger amplicon in *G. anatis*
8 (approx.1080 bp) was slightly smaller in *G. genomospecies 1* and *G. genomospecies 2*
9 (approx. 1030 bp). The internal amplification control based on the *rpoB* gene generated an
10 amplicon of approx. 520 bp. **B)** PCR amplicons from *Gallibacterium anatis* biovar *anatis*
11 (F149^T), *G. anatis* biovar *haemolytica* (12656-12), *G. genomospecies 1* (CCM5975), *G.*
12 *genomospecies 2* (CCM5976) and *Pasteurella multocida* subsp. *multocida* (NCTC10322^T).
13 The internal amplification control based on amplification of the β -actin gene in trout
14 (*Oncorhynchus mykiss*) DNA generated an amplicon of approx. 570 bp. The O'Generuler 1
15 Kb DNA ladder was used as size marker.

1 **Table 1.** Bacterial reference strains included in this investigation.

Strain	Taxon	Biovar	Origin	Host	Country ^a
F149 ^T	<i>Gallibacterium anatis</i>	-	Intestine	Duck	DK
10672-6 Salp.	<i>G. anatis</i>	1	Salpingitis	Chicken	DK
12158-5 Salp.	<i>G. anatis</i>	3	Salpingitis	Chicken	DK
36961/S15	<i>G. anatis</i>	3	Unknown lesion	Chicken	DK
10672-9	<i>G. anatis</i>	4	Salpingitis	Chicken	DK
12656-12 Liver	<i>G. anatis</i>	4	Septicaemia	Chicken	DK
Gerl.2396/79	<i>G. anatis</i>	4	Unknown lesion	Chicken	G
Gerl.2740 A.salp.	<i>G. genomospecies 1</i>	5	Septicaemia	Pigeon	G
CCM5975	<i>G. genomospecies 1</i>	5	Respiratory tract	Chicken	Cz
CCM5974	<i>G. genomospecies 1</i>	8	Liver	Chicken	Cz
Gerl.3191/88	<i>G. genomospecies 2</i>	8	Unknown lesion	Chicken	G
CCM5976	<i>G. genomospecies 2</i>	9	Oviduct	Chicken	Cz
36961-S7	<i>G. anatis</i>	11	Trachea	Chicken	DK
Gerl.2737/S3/89	<i>G. anatis</i>	11	Unknown lesion	Turkey	G
BJ3453-2	<i>G. anatis</i>	12	Unknown	Bovine	DK
42447 Liver 2	<i>G. anatis</i>	12	Septicaemia	Chicken	DK
IPDH697-78	<i>G. anatis</i>	15	Unknown	Chicken	G
Gerl.3348-80	<i>G. anatis</i>	17	Airsacculitis	Goose	G
Gerl.3076/88	<i>G. anatis</i>	17	Unknown lesion	Duck	G
BK3387-2	<i>G. anatis</i>	18	Faeces	Bovine	B
Gerl.444/S5/89	<i>G. anatis</i>	18	Septicaemia	Budgerigar	G
20558-3 Cloaca	<i>G. anatis</i>	19	Cloaca	Goose	DK
CCM5995	<i>G. anatis</i>	20	Unknown	Chicken	Cz
4224 A. salp.	<i>G. anatis</i>	22	Unknown	Unknown	G
220-S1-89	<i>G. anatis</i>	24	Unknown	Unknown	G
NCTC4189 ^T	<i>Actinobacillus lignieresii</i>		Throat gland	Cow	UK
ATCC29546 ^T	<i>Avibacterium gallinarum</i>		Sinus infraorbitalis	Chicken	G
NCTC10370 ^T	<i>Bibersteinia trehalosi</i>		Septicaemia	Lamb	UK
NCTC9380 ^T	<i>Mannheimia haemolytica</i>		Unknown	Sheep	UK
P737	<i>M. glucosida</i>		Nose	Sheep	USA
UT18	<i>M. glucosida</i>		Nose	Sheep	Scotland
NCTC10322 ^T	<i>Pasteurella multocida ssp. multocida</i>		Unknown	Pig	Can
NCTC10204 ^T	<i>P. multocida ssp. gallicida</i>		Unknown	Bovine	UK
NCTC11411 ^T	<i>P. langaa</i>		Respiratory tract	Chicken	DK
101	<i>Volucribacter psittacidae</i>		Salivary gland	Parrot	G
NCTC11414	Bisgaard Taxon 2		Salpingitis	Duck	DK
S/C1101	Bisgaard Taxon 14	1	Lung	Turkey	UK
F75	Bisgaard Taxon 22		Trachea	Chicken	DK
F66	Bisgaard Taxon 26	1	Conjunctivitis	Duckling	DK
F97	Bisgaard Taxon 26	2	Trachea	Duckling	DK
HPA106	Bisgaard Taxon 32		Pneumonia	Sparrow Hawk	DK
B301529/00/1	Bisgaard Taxon 40		Respiratory tract	Seagull	Scotland
4237/2sv	<i>Riemerella anatipestifer</i>		Pharynx	Duckling	DK
G9	<i>Salmonella Gallinarum</i>		Septicaemia	Chicken	UK
14R525	<i>Escherichia coli</i>		Intestine	Human	Unknown
726-82 ^T	<i>Coenonia anatina</i>		Unknown	Duck	G
CUG23171 ^T	<i>Ornithobacterium rhinotracheale</i>		Respiratory tract	Turkey	UK

2 ^aB: Belgium, Can: Canada, Cz: Czech republic, DK: Denmark, G: Germany, UK: United kingdom. ^T Type strain

Figure 1

