

First report of biovar 6 in birds immunized against *Gallibacterium anatis* in poultry farms located in Sonora, México

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Abstract

Identification of the predominant circulating biovars is important for controlling *Gallibacterium anatis* infections in poultry because efficient protection is based on the use of biovar-specific immunogens. Twenty-three isolates of *Gallibacterium anatis* were recovered from commercial laying hens with respiratory and reproductive clinical signs. Bacteria were identified as *G. anatis* based on morphology, biochemistry, and PCR testing. Biochemical analyses identified the isolates as biovar 6 which had not previously been reported in Sonora, México. Therefore, the recent finding of biovar type 6 could explain clinical presentations of this disease in immunized commercial laying flocks in Sonora.

Keywords: *Gallibacterium anatis*, Biovar, biotyping, PCR.

Introduction

Infectious diseases of poultry, especially bacterial diseases of the respiratory system, cause significant losses in poultry production due to the increased mortality of birds and high treatment costs.¹ Over the last 10 years, the poultry industry in Sonora noted a considerable decrease in egg production. It was established that this decrease was associated with *Pasteurella haemolytica-like* bacteria, but since these bacteria were considered normal gut flora, this line of investigation was not pursued further.² Some years later, genetic changes in these bacteria were associated with an increase in virulence. This discovery led to the establishment of a new

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genus within the family *Pasteurellaceae*, and the classification of these bacteria as *Gallibacterium anatis* biovar haemolytica.³

Gallibacterium anatis is common in microbiological analyses and affects both the respiratory and reproductive systems. Infection may cause a decrease in egg production and produce a variety of lesions, including follicular atrophy, oophoritis, septicaemia, misshapen follicles, peritonitis, enlarged kidneys, enteritis, congestion, and oedema of the respiratory tract.^{2,4,5} Mirle *et al.*⁶ established that *Gallibacterium anatis* is also associated with variation in egg size, eggshell formation, and reductions of 4 % to 20 % in egg production. *Gallibacterium anatis* represents both a significant health risk and an economic challenge for the Mexican poultry industry.

In the present study, *Gallibacterium anatis* isolates were isolated from commercial layer hens in the southern part of the state of Sonora to determine the biovars in the region. These results will be used to implement improved control programmes based on the use of the correct immunogens. Classification of *Gallibacterium anatis* can be based on biochemical reactions.⁷ Thirteen biovars of *G. anatis*, 7 of which have been reported in Mexico (1, 2, 4, 5, 8, 16 and U), can be identified using the approach developed by Jaworski *et al.*⁷ Bisgaard developed a biochemical classification system with 24 biovars, thereby providing a more specific classification system.^{8,9} Based on the latter classification system, biovars 1, 4, 11, 12, 13, and 22 are present in Mexico, with occasional reports of biovars 3, 7, 15, 16, 17, 18, 19, 20, and 24. Biovars 1 and 4 have been reported in the southern areas of the state of Sonora.^{2,3}

Material and methods

Sampling

A total of 600 hens, twenty birds each from 30 farms, with respiratory and reproductive signs and medical histories were collected. Sampling and necropsies were performed following the procedures described elsewhere.^{10,11} This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all efforts were made to minimize suffering.

Isolation and identification of *Gallibacterium anatis*

Swab samples collected from the turbinates, trachea, lungs, cleft palate, liver, spleen, kidneys, follicles, and peritoneum were cultivated on blood agar (BD Bioxon) containing 5 % sterile defibrinated bovine blood and on MacConkey agar (BD Bioxon). The samples were incubated at 37 °C for 18 to 24 hours in a microbiological incubator (Shel Lab 1535).^{12,13} For morphological identification of bacteria, colonies with a diameter of 1 to 2 mm that were circular, convex, and translucent grey, were purified and selected based on Christensen's criteria.³ Bacteria were stained with Gram stain, visualized using an Olympus microscope CX41, and identified as gram-negative coccobacilli.¹⁴⁻¹⁶ Biochemical procedures for the identification of bacteria were then performed using cultures that were < 24 hours old, and carbohydrate fermentation, enzyme production, and the production of certain metabolites were analysed using MicroScan Renok™ for gram-negative

bacteria. Following the manufacturer's recommended procedure, inoculated plates were incubated at 37 °C for 24 hours (Shel Lab 1535). Because of the absence of *Gallibacterium anatis* in the MicroScan database, manual interpretation was used, i.e. in each well, a specific colour change was evaluated as a surrogate for a metabolic reaction. In addition, biochemical analyses for other carbohydrates (glucose, maltose, trehalose and D-xylose) were performed (BD Bioxon) to complement the biochemical tests and identify the bacteria. All biochemical tests were performed twice. In addition, the oxidase test was performed for the presence of the oxidase enzyme using a dipstick (Bactident® Oxidase). Isolate typing was based on previous reports of the carbohydrate fermentation of (+) L-arabinose, (+) D-xylose, m-inositol, (-) D-sorbitol, maltose, trehalose, and dextrin, that differentiated the 24 registered biovars (Table 1).^{3,8}

Thereafter, to confirm bacterial identity, polymerase chain reactions PCR targeted to amplify specific gene segments of *Gallibacterium anatis* were performed. Bacterial DNA was extracted using a QIAamp DNA Mini Kit (51306, QIAGEN) following the manufacturer's instructions. The DNA used in the (PCR) was adjusted to a concentration of 50 µg/µl. The primers and oligonucleotides were designed based on the 99 sequences of 16S rRNA from Gen Bank.¹⁷ The primer 1133fgal (5'-TAT-TCTTTGTTACCARGG) is specific to *Gallibacterium* and does not amplify DNA from other *Pasteurellaceae* within the chosen PCR conditions. For inverse amplification, the primer of the 23S rRNA gene with the sequence 114r (5'-GGTTCCCCATTCGG) was selected.^{18,19} PCR was performed with the default settings of the thermocycler (ThermoFisher Gene 9700) and with 2.5 µl of Mg 10X buffer, 0.75 µl of MgCl₂ (50 mM), 0.5 µl of dNTPs mix (10 mM), 0.25 µl each of F primer and R primer (25 µM), 0.25 µl of Taq (5 U/µl), 18.5 µl of RNase-free H₂O, and 2 µl of DNA from each of the samples. Denaturation was performed for 4 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds each, and then the alignment was performed for 1 min at 54 °C and 2 min at 72 °C, followed by a final extension of 72 °C for 10 min. The amplification products (790 bp and 1080 bp for the genus *Gallibacterium*) were examined by the separation of PCR products during electrophoresis on 1.6 % agarose gel stained with ethidium bromide.^{18,20} The amplicons were visualized on a UV transilluminator (UV-25 Macro Vue, Hoefer).

Results and discussion

In this study, a known isolate from previous studies was included as a control. Isolates of *Gallibacterium* spp. were identified by their morphological and haemolytic characteristics as described by Christensen *et al.*³ Although 600 samples were analysed, the recovery of *Gallibacterium* spp. was not possible in all cases, perhaps because hens were previously immunized against this organism. These results support the idea that this organism causes respiratory and reproductive disease because 60 % of the isolates of *Gallibacterium anatis* were obtained from lungs: 30 % in tracheae and 30 % in follicles.

The classification of biovars via biochemical tests^{3,8,9} showed that 41 % of the bacteria were biovar 1, and 59 % were biovar 6 (Table 1). As opposed to previous reports, biovar 4 was not recovered in this study.² In the extraction process, an average of 20.5 µg/µl of DNA and a DNA purity ratio of 1.8 were achieved (Table 2).

Table 1. Biochemical tests on each bacterial isolate.

Biovars of isolated strains from <i>Gallibacterium anatis</i>														
Biovar	Biochemical Tests												Hemolysis	# Samples
	Maltose	Dextrin	Trehalose	Xylose	Glucose gas	Sucrose acidity	Sorbitol	Arabinose acidity	Inositol acidity	Oxidation fermentation of glucose	Nitrate	Oxidase		
6	+	+	+	+	+	+	+	-	-	+	-	+	+	1
1	+	+	+	+	+	+	+	-	+	+	+	+	+	2
6	+	+	+	+	+	+	+	-	-	+	-	+	+	3
6	+	+	+	+	+	+	+	-	-	+	+	+	+	4
1	+	+	+	+	+	+	+	-	+	+	+	+	+	5
6	+	+	+	+	+	+	+	-	-	+	+	+	+	6
1	+	+	+	+	+	+	+	-	+	+	-	+	+	7
1	+	+	+	+	+	+	+	-	+	-	-	+	+	8
6	+	+	+	+	+	+	+	-	-	+	-	+	+	9
1	+	+	+	+	+	+	+	-	+	-	+	+	+	10
6	+	+	+	+	+	+	+	-	-	-	+	+	+	11
6	+	+	+	+	+	+	+	-	-	+	+	+	+	12
1	+	+	+	+	+	+	+	-	+	-	+	+	+	13
6	+	+	+	+	+	+	+	-	-	+	+	+	+	14
1	+	+	+	+	+	+	+	-	+	+	+	+	+	15
6	+	+	+	+	+	+	+	-	-	-	-	+	+	16
6	+	+	+	+	+	+	+	-	-	-	+	+	+	17
6	+	+	+	+	+	+	+	-	-	-	+	+	+	18
6	+	+	+	+	+	+	+	-	-	+	+	+	+	19
1	+	+	+	+	+	+	+	-	+	+	-	+	+	20
1	+	+	+	+	+	+	+	-	+	-	+	+	+	21
6	+	+	+	+	+	+	+	-	-	+	+	+	+	22
1	+	+	+	+	+	+	+	-	+	+	+	+	+	23

Table 2. Concentration and purity of the extracted DNA from each bacterial isolate of *Gallibacterium anatis*.

DNA concentration from <i>Gallibacterium anatis</i> strains					
# Samples	µg/ml	DNA Purity	# Samples	µg/ml	DNA Purity
1	15	1.73	13	25	1.73
2	35	1.83	14	5	1.54
3	22.5	1.83	15	22.5	1.73
4	32.5	1.9	16	20	1.76
5	25	1.82	17	17.5	1.74
6	20	1.87	18	12.5	1.94
7	32.5	1.78	19	17.5	1.66
8	20	1.79	20	22.5	1.73
9	20	1.68	21	17.5	1.61
10	20	1.9	22	15	1.7
11	20	1.81	23	15	1.63
12	20	1.69	AVERAGE	20.5	1.8

Likewise, the presence of *Gallibacterium anatis* was confirmed by *G. anatis*-specific PCR for 16S rRNA and 23S rRNA.¹⁹ As reported elsewhere,^{3,19,21} these procedures rule out other members of the family Pasteurellaceae. The amplification products of the isolated bacteria were 790 bp and 1080 bp long, thereby confirming the identity of the 23 isolates as *Gallibacterium anatis* (Figure 1).

Diagnostics performed in the state of Sonora between 2009 and 2011 identified biovar 6 and biovar 1 in birds immunized with commercial products containing biovar 1 and biovar 4. These findings suggested that the outbreak might have occurred because biovar 6 was absent in the commercial immunogen.² Based on these results, it is recommended that immunogens include biovar 6 to prevent future outbreaks.

The identification of strains of *G. anatis* is often burdensome because of the difficulty in interpreting phenotypic data.^{3,8} For this reason, PCR is highly useful in the diagnosis of *Gallibacterium anatis*. Although the use of molecular methods is constrained by the high cost of PCR, biotyping is an important tool in outbreak investigations. Tegtmeier *et al.*²¹ demonstrated that bacterial identification through conventional methods is less sensitive than either immunohistochemistry or PCR. This highlights the importance of having alternative diagnostic tools available for *G. anatis* in order to rule out other gram-negative pathogens associated with similar clinical signs, e.g., *E. coli*, and start specific treatment.^{8,19}

Conclusions

Overall, the results of this study showed the presence of *Gallibacterium anatis* in association with respiratory and reproductive problems in populations of commercial laying hens in southern Sonora. Additionally, for the first time in Mexico,

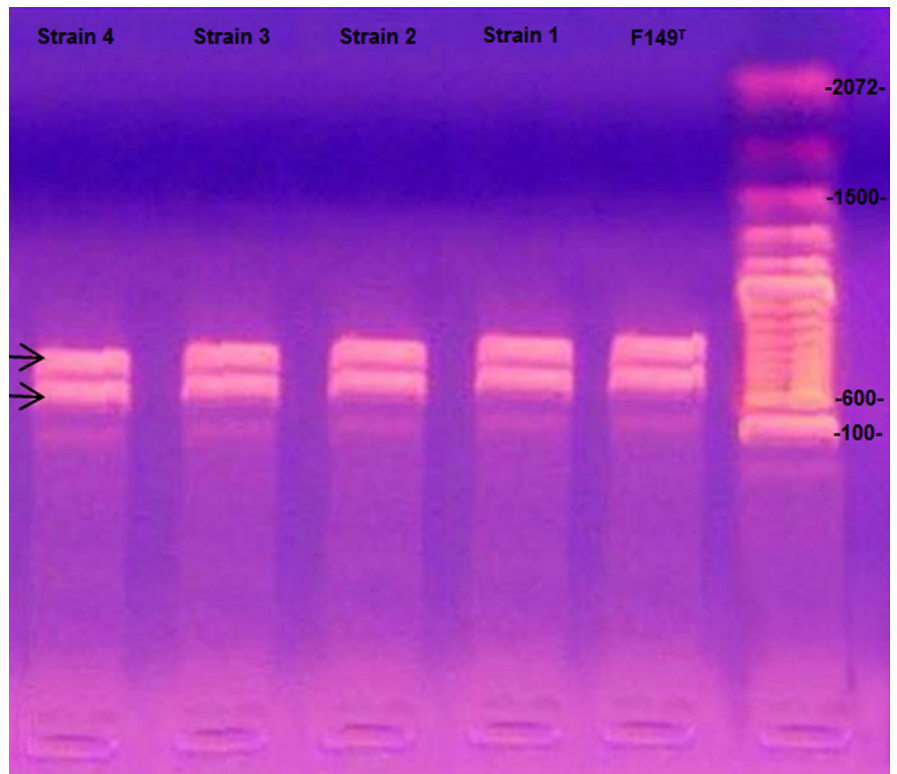


Figure 1. PCR amplification gene, *Gallibacterium anatis*.

G. anatis biovar 6 was detected. The presence of this new biovar will provide a basis for understanding pathogenesis and immunity, and it may explain the clinical presentation of *Gallibacterium anatis* in immunized birds.

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Conflicts of interest

The authors declared having no conflict of interest in this study.

Author contributions

Reyna Fabiola Osuna Chávez: Performed the study and wrote the manuscript.

Ramón Miguel Molina Barrios: Performed the study and wrote the manuscript.

Juan Francisco Hernández Chávez: Molecular analysis

Jorge Robles Mascareño: Microbiological analysis

Jesús Gabriel Adrián Icedo Escalante: Sampling.

Martín Acuña Yanes: Sampling and processing of samples.

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