**ABSTRACT**

In our investigation phenotypic and genotypic characterization were carried out for detection of *Gallibacterium anatis* in chickens and ducks that suffered from septicemia, perheptitis, percarditis, trachitis and penumonia. Prevalence of *Gallibacterium anatis* was 24% and 26% from diseased chickens and ducks respectively. The biochemical investigations differentiated the isolated strains into two biovars *Gallibacterium anatis biovar haemolytica with a percentage* 24 % and *Gallibacterium anatis biovar anatis* 76 %. *Gallibacterium anatis biovar haemolytica* represented 30.7% in ducks and 16.6% in chickens while *Gallibacterium anatis biovar anatis* was represented 69.2% in ducks and 83.3% in chickens. Antimicrobial susceptibility test insured the multidrug resistant of *Gallibacterium anatis* isolates. PCR technique confirms isolation percentage. Also virulence genes were examined like cytotoxic genes (gtx A1 and A2 and its results were relevant to the phenotypic virulent characteristics in addition to fimberial gene (flfA.). In conclusion some virulent genes like gtxis responsible for evoking the phenotypic character of strain such as hemolysis.

**Key words:** Gallibacterium anatis, Multidrug resistant, gtx gene, flf gene, poultry.

**INTRODUCTION**

*Gallibacterium* is a genus in the Gram negative bacteria that is commonly associated with poultry (Mushin et al., 1980 and Bisgaard et al., 2009). Also it was recently established within the family of Pasteurellaceae (Christensen et al., 2003). The genus contains avian bacteria formerly known as *Pasteurella haemolytica* like, *Actinobacillus salpingitidis* or *Pasteurella anatis* and currently includes the species *Gallibacterium anatis* and *Gallibacterium genospecies* 1 and 2. Two biovars are described within G.anatis, a haemolytic biovar haemolytica and a non haemolytic biovar anatis. *Gallibacterium* spp. Can be isolated from a great variety of birds such as chickens, turkeys, ducks, geese, psittacine bird’s, partridges and guinea fowl (Addoan Mohan, 1984 and Bisgaard, 1993). Some authors like Bojesen et al. (2003) mentioned that G. anatis is a common part of the normal flora of both the upper respiratory tract and lower genital tract of chickens and other avian species. G. anatis divided into two biovars: the b-haemolytic biovar haemolytica and the non-haemolytic biovar anatis. The ability to lyse red blood cells is a prominent phenotype of pathogenic G. anatis isolates and the production of haemolysin is a likely virulence factor. (Christensen et al., 2003) *Gallibacterium anatis* has recently been recognized as a major cause of lesions in the reproductive tracts of egg layers (Neubauer et al., 2009), causing a drop in egg production and increased mortality (Bojesen et al., 2008). Multiple-drug resistance (Bojesen et al., 2011) and a substantial antigenic diversity (Vazquez et al., 2006) make it difficult to prevent the negative effects of *Gallibacterium anatis* using traditional anti microbial agents and vaccine. In field study performed in Denmark by Bojesen et al. (2003), demonstrated that the biosecurity level influenced the prevalence of *Gallibacterium* spp., with a lower level of biosecurity resulting in a more frequent detection of these pathogens. Fimbriae have been intensively studied, not only because they are important virulence factors of bacteria, but also because they are among the most widely used targets for the development of interventions such as vaccines (Seavone et al., 2011; Hur and Lee 2012; Wang et al., 2013). Moreover RTX-toxins are important virulence factors and responsible for haemolytic and leukotoxic activity in bacteria related to *Gallibacterium* (Frey and Kuhnert, 2002).

The aim of the work is isolation of the *Gallibacterium* spp. Associated with the diseased cases also focusing in detection of some virulent genes that may increase the signs and deaths. Finally
studying the antibiotic susceptibility pattern to help Effective control measures are required to mitigate the economic impact on the poultry industry.

MATERIALS and METHOD

Collections of 100 samples of chickens and ducks suffering from respiratory signs (50 from each species) shown septicaemia, perihepatitis, pericarditis, trachitis and pneumonia. The examined Samples included: trachea, liver, heart and lung.

A-Bacterial investigations of Gallibacterium anatis:
1 - Trypticase soy agar enhanced by addition of 0.05% yeast extract and 5% newborn calf serum. (Sandhu and Richard, 1997).

2- Brain heart Infusion medium (Li et al., 2011).

3- Blood agar: within 24 hour, which is characterized as follows: circular, raised colonies with entire margin, shiny and semi-transparent with a β hemolytic zone. Such colonies were regarded as suspicious of Gallibacterium. Suspected colonies were subcultured on blood agar to obtain pure cultures. (Neubauer et al., 2009).

4- Brain heart Infusion broth:
A- Propagation and maintenance of bacterial cultures for improved growth (Zepeda et al., 2009).
B- Preservation (Bojesen et al., 2003).

Seven hundred microlitres of Brain heart Infusion broth were mixed with 300μl sterile glycerol 50% and stored at – 80 °C until further use.

5- India ink

6- API 20 for differentiation of Gallibacterium anatis According to (Florence et al., 2008).

Table 1: API 20 for detection of Gallibacterium anatis biovar heamolytica and biovar anatis (Olsen et al., 2005).

<table>
<thead>
<tr>
<th>Indole</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Motility</th>
<th>Sucrose</th>
<th>Urease</th>
<th>ODC*</th>
<th>Trehalose</th>
<th>Sorbitol</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>D</td>
<td>D</td>
<td>chicken</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>D</td>
<td>Duck</td>
</tr>
</tbody>
</table>

ODC*: Ornithine decarboxylase. (+): 90% or more of the strains are positive
D: 11-89% of the strains are positive according to the criteria in Bergeys Manual of systematic Bacteriology (-): 10% or less of the strains are positive.

7- Antimicrobial sucesexptalibility test for Gallibacterium anatis According to (Matthew et al., 2009).

8- Congo red binding test for detection of virulence Gallibacterium anatis according to (Berkhoff and Vinal, 1986).

B- Conventional PCR technique.

Extraction:
Gallibacterium anatis DNA was extracted using commercially available kit, QIAamp DNA Mini Kit, Catalogue no.51304.

PCR Reaction:
The different primers used in this study are described in Table (2).

Table 2: Oligonucleotide primers and sequences encoding for detection of Common gene, flfA gene and gtxA.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Primer sequence(5'-3')</th>
<th>Size of Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S RNA &amp; 23S RNA</td>
<td>1133F</td>
<td>TATTTTTGGTACCACCG</td>
<td>1032</td>
<td>Bojesen et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>114R</td>
<td>GGTTCCTCCATTCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flfA</td>
<td>1162 F</td>
<td>CACCATGGGTGCAATTGCGGATGATGCC</td>
<td>538</td>
<td>Bager et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>1162 R</td>
<td>TATTCGTATCGTGATGATGATGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GalNtxR</td>
<td>GGATAATCGGTGCGGCTTTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

According to morphological characters and biochemical reactions the Gallibacterium was 24% (12/50) in diseased examined chickens and 26% (13/50) in diseased examined ducks.

Differentiation between the hemolytic and non-hemolytic Gallibacterium was defined using the blood agar which was characterized as follows:

circular, raised colonies with entire margin, shiny and semi-transparent with a β hemolytic zone. The phenotypical studies insured that all isolates of Gallibacterium were virulent 100% when detected by congo red test, API 20™ used for differentiation of Gallibacterium anatis into Gallibacterium anatis biovar haemolytica and Gallibacterium anatis biovar anatis as detailed characters in Table (1) also results of classification are described in Table (3).

Table 3: Results of classification of gallibacterium anatis into two biovars, β haemolytic biovar haemolytica and non- haemolytic anatis on blood agar and API 20.

<table>
<thead>
<tr>
<th>Type of bird</th>
<th>β haemolytic gallibacterium</th>
<th>non- haemolytic anatis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>Chickens</td>
<td>2/12</td>
<td>16.6%</td>
</tr>
<tr>
<td>Ducks</td>
<td>4/13</td>
<td>30.7%</td>
</tr>
<tr>
<td>Total</td>
<td>6/25</td>
<td>24%</td>
</tr>
</tbody>
</table>

Antimicrobial susceptibility test resulted that Gallibacterium anatis were characterized by multidrug resistant. Gallibacterium anatis isolates were resistant 100% to Ampicillin, Gentamycin 10, Erytheromycin 15, Ciprofloxacin 5, Chloramphenicol 30, linconspectinomyacin, Doxycycline, Tetracycline Streptomycin, Naldixie acid, Enerfloxacin, Neomycin and Lincomycin, while the resistant percentage was 78.5% to Sulphamethazole and Trimethoprim, 57.1% to clostin sulphate and 50% to Pencillin.

The isolation results were confirmed by PCR technique using 16S RNA and 23S RNA common primer for the Gallibacterium anatis Also the results for the two examined virulent gene for GtxA (cytolytic- hemolytic gene) and flf A fimberial gene are described in Table (4). Figure 1 and 2, Showed the positive amplification for the two examined virulence genes.

Table 4: PCR results for the different examined genes.

<table>
<thead>
<tr>
<th>Type of bird</th>
<th>chickens</th>
<th>Ducks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR identification of isolated isolates by 16S RNA&amp;23S RNA (common species specific)</td>
<td>12/12(100%)</td>
<td>13/13(100%)</td>
</tr>
<tr>
<td>Detection of virulence gene by using PCR</td>
<td>7/12 (58%)</td>
<td>5/13(38.4%)</td>
</tr>
<tr>
<td>1- Toxin gene GTX A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2- Flagellar gene</td>
<td>6/12(50%)</td>
<td>2/13(15.38%)</td>
</tr>
</tbody>
</table>
Fig. 1: positive amplification of *G. anatis* isolates for gtxA1 gene from left to right, lane (M) ladder (QIAGEN, Gmbh) (100+ bp), the positive amplification of gtx A1 gene at 925bp

Fig. 2: positive amplification of *G. anatis* isolates for flfA gene, from left to right, lane (M) 100 bp ladder (QIAGEN, Gmbh) (100-600 bp), (negative control), (positive control), the positive amplification of flfA gene at 538bp.

### DISCUSSION

The global demand for meat and animal products is raising as the world population and income increases. Poultry meat and eggs are considered very important and highly sustainable components of the future global diet (AVEC, 2011). Pasteurellaceae are bacteria with an important role as primary or opportunistic, mainly respiratory, pathogens in domestic and wild animals. Some species of Pasteurellaceae cause severe diseases with high economic losses in commercial animal husbandry and are of great diagnostic concern. (Florence et al., 2008).

In our investigation *Gallibacterium anatis* was diagnosed in chickens and ducks that were suffered from septicemia, pericarditis, trachitis and pneumonia. The prevalence of *Gallibacterium anatis* was 24% in examined chickens and 26% in examined ducks. This agrees with Neubauer et al. (2009) who reported isolation of *Gallibacterium* in pure cultures of samples from birds with various pathological lesions. Evidently, the organism is capable of causing serious, systemic infections affecting multiple organ systems but the mechanisms of pathogenesis remain obscure. We differentiated the isolates into *Gallibacterium anatis* biovar *heamolytica* 24 % and *Gallibacterium anatis* biovar *anatis* 76 %. *Gallibacterium anatis* biovar *heamolytica* was represented 30.7% in ducks and 16.6% in chickens while *Gallibacterium anatis* biovar *anatis* was represented 69.2% in ducks and 83.3% in chickens. These results were previously explained by (Addoand Mohan, 1984; Bisgaard, 1993) that the genus contains avian bacteria formerly known as *Pasteurella haemolytica* like, *Actinobacillus salpingitidis* or *Pasturella anatis* and currently includes the species *Gallibacterium anatis* and *Gallibacterium* genomospecies 1 and 2. Two biovars are described within *G. anatis*, a haemolytic biovar *heamolytica* and a non haemolytic biovar *anatis*. The haemolytic activity of *Gallibacterium* spp. was very prevalent in the Danish chicken production system of low to moderate biosecurity level, as recorded by Anders et al. (2003). Indicating that lesser biosecurity is a major risk factor for obtaining a *Gallibacterium* infection. But this result was in contrary to that obtained in our study in which the prevalence in ducks was more than that of chickens. In our investigation, isolated *Gallibacterium anatis* were resistant to *Gallibacterium anatis* isolates were resistant 100% to Ampicillin, Gentamicin 10, Erythromycin 15, Ciprofloxacin 5, Chloramphenicol 30, lincomycin, Doxycycline, Tetracycline, Streptomycin, Nalixic acid, Enorflaxin, Neomycin and Lincomycin, while the resistant percentage was 78.5% to Sulphamethazole and Trimethoprim, 57.1%
to clostin sulphate and 50% to Pencillin. These findings were consistent with those of Bojesen et al. (2011) which observed 65% multidrug resistance in 74% of the field strains and only two strains were susceptible to all compounds. Most prominently, resistance to tetracyclines and sulfamethoxazole was observed in 92% and 97% of the field strains, respectively. For comparison, these figures were 67% and 42% respectively for the reference strains. The results were close to those obtained by Jones et al. (2013) which demonstrated Gallibacterium anatis to be completely resistant to novobiocin, tylosin, lincosamide and tetracycline antimicrobials with moderate to high sensitivity to sulfonamides, fluoroquinolones and florfenicol. This intermediate sensitivity to spectinomycin and erythromycin and variable resistance to lactam and aminoglycoside antimicrobials.

To represent possible antigenic variation between Gallibacterium fimbrial clusters, all Gallibacterium CU fimbriae were classified into five types based on fimbrial subunit phylogeny. All fimbrial clusters in which a previously described FlfA homolog was detected and named as flf, and those in which major fimbrial subunits were assigned to the different phylogenetic groups than flfA were named as flf1, flf2, flf3 and flf4. Moreover, many researches mentioned the fimbrial structure of Gallibacterium that fimbrial subunit protein (flfA) was identified as a promising candidate that may be used to vaccinate laying hens (Bager et al., 2013; Bager et al., 2014). In our study, the flfA gene represented 50% in the isolates of chicken and 15.38% from duck samples. Kudirkienė et al., 2014 cleared that from the five defined CU fimbrial clusters, the most common fimbrial cluster was fflA found in 74% Gallibacterium genomes, followed by flf2 and flf3 detected in 65% and 52% of the genomes, respectively. Few virulence factors of G. anatis have been described. One key virulence factor of G. anatis is its RTX-like toxin named gtxA and its associated secretion system (Kristensen et al., 2010 and 2011). In our research, the gtxA gene was detected in 7/12 (58%) and 5/13 (38.4%) from chicken and duck samples respectively. Also, the isolates that phenotypically lack the ability to hemolyze the RBCs are lacking the RTX toxin producing gene. These results are correlated to the results of hemolytic activity of the isolates which have the B hemolytic glycolytic genes as noted in some researches that the RTX-toxins are important virulence factors and strains lacking these genes have reduced virulence (Tascon et al., 1994; Jansen et al., 1995; Tatum et al., 1998). The virulence mechanisms possessed by G. anatis, and its role as a pathogen, have not been fully elucidated. The prototypic virulent G. anatis strain 12656-12 was recently sequenced. Analysis of this sequence identified a RTX-like toxin, gtxA, which contributes to G. anatis’ virulence for chickens, and has cytotoxic activity (Kristensen et al., 2010). Furthermore, gtxA was identified and was found to be disrupted in non-hemolytic strains of G. anatis (Kristensen et al., 2011). Other virulence associated traits among G. anatis strains have been identified, such as protease production and hemagglutination, but the underlying genetic traits responsible for these phenotypes have not yet been determined. It has also been proposed that G. anatis isolates vary in their virulence potential (Zepeda et al., 2010 and 2009), and amplified fragment length polymorphism (AFLP) has revealed that there is substantial genetic diversity among the Gallibacterium isolates dominating among and between successive flocks (Bojesen and Shivaprasad., 2007).

REFERENCES


