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GALLIBACTERIUM ANATIS INFECTION IN CHICKENS AND DUCKS

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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 starins *Gallibacterium anatis biovar heamolytica with a percentage* 24 % and *Gallibacterium anatis biovar anatis* 76 %. *Gallibacterium anatis biovar heamolytica* 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 *gtx*is 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 genomospecies* 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 (Addoand Mohan, 1984 and Bisgaard, 1993). Some authers 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. anatisis 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 alower 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 (Scavone *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 septicemia, perihepatits, pericarditis, trachitis and penumonia. 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 heamolytic 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).

Indole	oxidase	catalase	Motility	Sucrose	urease	ODC*	Trehalose	Sorbitol	host
-	+	+	-	+	-	-	D	D	chicken
-	+	+	-	+	-	+	D	+	Duck

ODC*: Ornithine decarboxylase, **(+):** 90% 0r 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 suscessptalibity 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).

PCR amplification.
It was done in a 25 µl reaction containing 12.5 µl of Emerald Amp GT PCR master mix (2x premix), 1 µl of each primer (20 pmol conc.), 4.5 µl of PCR grade water, and 6 µl of template. The cPCR reactions were performed in a Biometra T3 thermal cycler. And the thermal profiles for the different genes were applied as discussed in the reference showed in Table (2).

The PCR products were separated by electrophoresis on 2% agarose gel and photographed by a gel documentation system (Alpha Innotech, Biometra).

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

Target gene	Primer	Primer sequence(5'-3')	Size of Amplicon (bp)	Reference
16SRNA & 23S RNA	1133F	TATTCTTTGTTACCACGG	1032	Bojesen *et al.* (2007)
	114R	GGTTTCCCCATTCGG		
flfA	1162 F	CACCATGGGTGCATTTGCGGATGATCC	538	Bager *et al.* (2013)
	1162 R	TATTCGTATGCGATAGTATAGTTC		
GTX –N terminal	GalNtxF	TGCGCAAGTGCTAAATGAAG-	925	Paudel *et al.* (2013).
	GalNtxR	GGATAATCGTTGCGCTTTG-		

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 heamolytic zone. The phenotypical studies insured that all isolates of *Gallibacterium* were virulent 100% when detected by congo red test, API 20^{TM} used for differentiation of *Gallibacterium anatis* into *Gallibacterium anatis biovar heamolytica* 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 ager and API 20.

Type of bird	β haemolytic gallibacterium		non- haemolyticanatis	
	Number	**%**	**Number**	**%**
Chickens	2/12	16.6%	10/12	83.3
Ducks	4/13	30.7%	9/13	69.2
Total	6/25	24%	19/25	76%

Antimicrobial susceptibility test resulted that *Gallibacterium anatis* were characterized by multidrug resistant. *Gallibacterium anatis* isoltates were resistant 100% to Ampicillin, Gentamycin 10, Erytheromycin 15, Ciprofloxacin 5, Chloramphenicol 30, lincospectinomycin, Doxycycline, Tetracycline Streptomycin, Naldixic 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 *Gtx*A (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.

Type of bird	**chickens**	**Ducks**
PCR identification of isolated isolates by 16S RNA&23S RNA (common species specific)	12/12(100%)	13/13(100%)
Detection of virulence gene by using PCR 1- Toxin gene GTX A1	7/12 (58%)	5/13(38.4%)
2- Flagellar gene	6/12(50%)	2/13(15.38%)

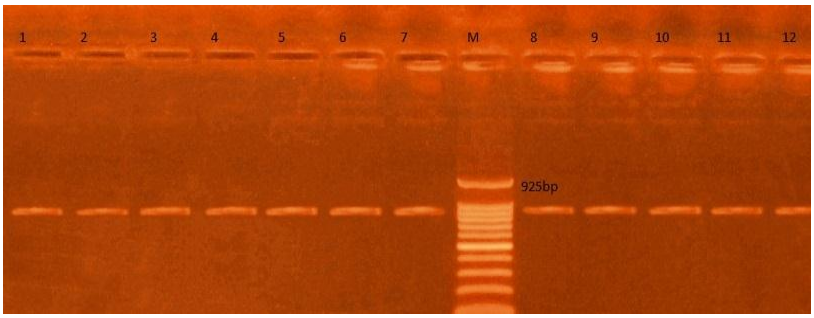

Fig. 1: positive amplification of *G. anatis* isolates for *gtx A1* gene from left to right, lane (M) ladder *(QIAGEN, Gmbh) (100+ bp),* the positive amplification of *gtx A1* gene at 925bp

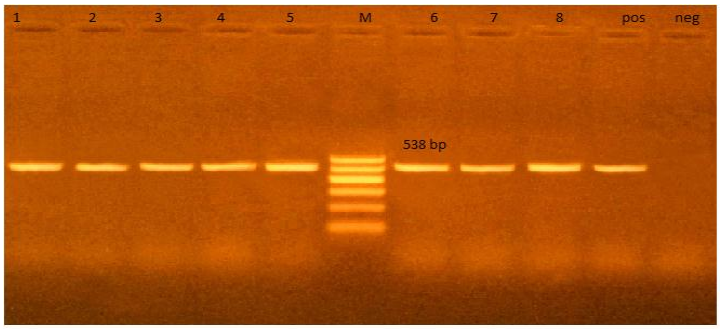

Fig. 2: positive amplification of *G. anatis* isolates for *flfA* gene, from left to right, lane (M) 100 bp ladder *(QIAGEN, Gmbh) (100-600 bp),* (negative control), (positive control), the positive amplification of *flfA* gene at 538bp.

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, periheptitis, pericarditis, trachitis and penumonia The prevelance of *Gallibacterium anatis* was 24% in examined chickens and 26% inexamined 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 biovaranatis* 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 *Pasteurella anatis* and currently includes the species *Gallibacterium anatis* and Gallibacterium genomospecies 1and 2. Two biovars are described within G.anatis, a haemolytic biovar haemolytica 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 results 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* isoltates were resistant 100% to Ampicillin, Gentamycin 10, Erytheromycin 15, Ciprofloxacin 5, Chloramphenicol 30, lincospectinomycin, Doxycycline, Tetracycline Streptomycin, Naldixic 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. These finding was near to that obtained by Bojesen *et al.* (2011) that tested 58 strains against 23 compounds of different classes multidrug resistance was observed for 65% of the field strains and only two strains were susceptible to all compounds. Most prominently, resistance to tetracycline and sulfa-methoxazole was observed in 92% and 97% of the field strains, respectively. For comparison these figures were 67% and 42% respectively for the reference strains. Our results were close to that obtained by Jones *et al*. (2013) that demonstrated *Gallibacterium anatis* almost complete resistance to novobiocin, tylosin, lincosamide and tetracycline antimicrobials with moderate to high sensitivity to sulfonamides, fluoroquinolones and florfenicol. There was intermediate sensitivity for 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 *flf*A homolog was detected and named as *flf*, and those in which major fimbrial subunits were assigned to the different phylogenetic groups than *flf*A were named as *flf*1, *flf*2, *flf*3 and *flf*4. Moreover Many researches mentioned the fimbrial structure of *Gallibacterium* that fimbrial subunit protein (*flf*A) 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 *flf*A gene represent 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 fimbrialcluster was *flf*1 found in 74% *Gallibacterium* genomes, followed by *flf*2 and *flf*3 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 *gtx*A and its associated secretion system (Kristensen *et al.*, 2010 and 2011). In our research the *gtx*A gene was detected in 7/12 (58%) and 5/13(38.4%) from chickens and ducks 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 has the *gtx* gene as insured in some researches that the RTX-toxins are important virulence factors and strains lacking these genes havereduced 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).

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عدوى الجاليبكتريوم انيتس في الدجاج والبط

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تم في دراستنا التوصيف المظهرى والجيني للجاليبكتريوم انتيس في الدجاج والبط التي كانت تعاني من تسمم دموى والتهابات فى غشاء القلب والكبد والقصبة الهوائيه وكانت نسبة حدوث الجاليبكتريوم انتيس في الدجاج المصاب والبط المصاب ٢٤% و ٢٦% علي التوالي. وبإستخدام اختبار انزيم البلمرة المتسلسل قد تم تأكيد نتائج العزل. وتم توصيف العترات المعزوله باستخدام التفاعلات البيوكيميائية وتقسيمها الي نوعين الجاليبكتريوم انتيس بايو ڤار هيموليتكا بنسبة ٢٤% وجاليبكتريوم انتيس بايو ڤار انيتس ٧٦% . جاليبكتريوم انتيس بايو ڤارهيموليتكا كانت نسبتها ٣٠,٧ فى البط و ١٦,٦ في الدجاج بينما جاليبكتريوم انتيس بايو ڤارانيتس ٦٩,٢ % في البط و ٨٣,٣ % في الدجاج. اكد اختبار الحساسية للمضادات الحيويه ان جاليبكتريوم انتيس يتميز بانه متعدد المقاومة للمضادات الحيويه. بالكشف عن جينات الضرواة لكلمن جين *gtx* وجين A *flf* . وجد ان بعض جينات الضرواة مثل *gtx* يكون مسئول عن التوصيف الظاهري مثل تحلل الدم.