

Gallibacterium anatis: An Emerging Pathogen of Poultry Birds and Domiciled Birds

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Rec date: Feb 09, 2016; Acc date: Mar 16, 2016; Pub date: Mar 18, 2016

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Abstract

Gallibacterium anatis though known since long as opportunistic pathogen of intensively reared poultry birds has emerged in last few years as multiple drug resistance pathogen causing heavy mortality outbreaks not only in poultry birds but also in other domiciled or domestic birds. Due to its fastidious nature, commensal status and with no pathognomonic lesions in diseased birds *G. anatis* infection often remains obscure for diagnosis. Poor understanding of its epidemiology, virulence factors and pathogenesis work on development of effective vaccine obscured its importance; however, it is difficult to get rid of *G. anatis* infection on affected poultry farms. The present review summarises the current knowledge on *G. anatis* and its infections.

Keywords: *Gallibacterium*; *Pasteurella anatis*; Egg drop; Mortality outbreaks; Multiple drug resistance; Vaccine

Introduction

Global meat production is predicted to rise by 1.6% over the outlook period, with poultry becoming the largest meat sector by 2020 [1]. *Gallibacterium anatis* infection is an emerging disease of poultry. Growing concern about *G. anatis* is its poorly understood growth kinetics, virulence markers, pathogenesis and vaccine(s) to control. *Gallibacterium anatis* (earlier known as *Pasteurella anatis*) is commensal in upper respiratory tracts and the lower genital tracts of healthy chickens [2,3]. It has been reported to be associated with bacteremia, oophoritis, follicle degeneration, salpingitis, peritonitis, hepatitis, enteritis, and respiratory tract diseases in chickens [4-8]. *Gallibacterium anatis* mostly affects intensively farmed poultry birds causing loss in production with heavy mortality in broiler chicken and drop in egg production in layers with increased mortality [9]. *Gallibacterium anatis* also reported to infect turkeys, geese, ducks, pheasants, partridges, budgerigars, peacock, cage birds, wild birds, cattle and pig [2,3,10-15]. Recently, it has also been associated with fatal bacteremia in immune-compromised patient [16]. *Gallibacterium anatis* causing disease in birds has been reported from all continents [2,4,12,17,18]. Its association with a variety of pathology makes it difficult to be diagnosed even after post-mortem in absence of pathognomonic lesion(s) and the disease is often confused with Fowl Coryza, New Castle disease and Bird Flu [12].

Gallibacterium anatis, a Gram-negative, rod shaped, non-motile, capsulated, facultative anaerobic bacteria was classified in family Pasteurellaceae by Pohl [11,12]. *Gallibacterium anatis* has two biovars i.e., a haemolytic biovar, Haemolytica and a non-haemolytic biovar, Anatis [12]. Currently, *G. anatis* and *G. genomospecies* 1, 2 [12], *G. genomospecies* 3 and *Gallibacterium* group V are the defined members of the genus *Gallibacterium* [11].

Though the infection of *G. anatis* is treatable with antibiotics, the frequency of treatment failure is an emerging and recurrent problem.

Multidrug resistant strains of *G. anatis* [5,19] have shown resistance to sulpha drugs, novobiocin, tylosin, clindamycin, tetracycline and penicillin [20-28]. Concerns have been shown for biosecurity measures towards control of disease, handling of pathogen and prevention of spread. *Gallibacterium* should be handled at biosafety level 2 (BSL-2) facilities, it has a little potential personnel and environmental risk, and however, more is too understood for its exact categorisation to some risk group.

Taxonomy of *G. anatis*

It belongs to phylum, Proteobacteria; class, Gammaproteobacteria; family, Pasteurellaceae; genus, *Gallibacterium* and has two biovars, haemolytica and anatis [12,29]. First time in 1950 the bacteria was isolated from cloaca of healthy chickens and was described as haemolytic "cloaca bacterium" by Kjos-Hansen. The meaning of *Gallibacterium* is 'bacterium of chicken'. Being similar to *Pasteurella* in several characters it was earlier known as *P. anatis*. The genus name *Gallibacterium* was first given by Bisgaard in the year 1982 on the basis of certain phenotypic characters used for identification of *Actinobacillus salpingitidis* and avian *P. haemolytica* [2,10,11,30,31]. Christensen et al. [32] established the genus *Gallibacterium* within the family of Pasteurellaceae based on 16s rRNA gene sequences. The genus includes the strains belonging to *G. anatis*, *G. genomospecies* [1-3,12] and un-named group V [11]. Taxon 1 designated as a third group of strains, by Bisgaard in 1982 [30] and named *Pasteurella anatis* [29], was also found closely related to *A. salpingitidis* and avian *P. haemolytica*.

Besides this, comparison of whole genome by protein profiling [33] and amplified fragment length polymorphism [34] uncover the unique properties of five groups and assigned the proposal of new name to these three novel species of *Gallibacterium* naming, *G. melopsittaci* sp. nov. (Type strain F450T 5CCUG 36331T 5CCM 7538T), *G. trehalosi* fermentans sp. nov. (Type strain 52/S3/90T 5CCUG 55631T 5CCM 7539T) and *G. salpingitidis* sp. nov. (Type strain F150T 5CCUG 15564T 5CCUG 36325T 5NCTC 11414T).

However, with DNA-DNA hybridization they could not be clubbed into one genus [11,35]. Confusion is still persistent in naming biovars and may be a continued debate in coming years.

Epidemiology

Gallibacterium have been reported from many European countries viz., Switzerland, Denmark, Germany, Austria [2,36-38], African countries viz., Nigeria [4], Asian countries including China, India and Japan [17], American countries viz., USA [18,39,40], and several other countries like Colombia, Taiwan, Norway, Australia, Syria, England, Sweden and Czech Republic and recently in India. The epidemiological outcome strongly depends upon strain, route of inoculation and secondary factor [9]. Host and environmental factors have been observed to nurture the infection of *G. anatis*. Host factors playing a role are hormonal influences [41-43], age [2,44], stress [15], and compromised immunological status [45]. Important environmental factors are seasonal changes [23], and cold stress [46] in the similar pattern reported for infections with other members of Pasteurellaceae [38,47,48].

Habitat and host range

Gallibacterium strains are known since long as common inhabitants of the respiratory tract and lower genital tract of healthy chickens [2,10,49]. *Gallibacterium anatis* has been isolated from chickens, ducks, geese, guinea fowl, turkeys, pheasants, psittacine birds, partridges, web-footed birds, cattle egrets and budgerigars [3,10,49]. Especially biovar haemolytica has also been isolated from healthy [2,3,49,50] as well as sick birds, cattle and pigs [10-12,30,42,51].

Cultural and biochemical characteristics

The family Pasteurellaceae comprises large group of facultative anaerobes, gram-negative, non-sporing, chemo-organotrophic and fermentative bacteria including the genus *Gallibacterium*. All *Gallibacterium* species strains are non-motile have rod-shaped or pleomorphic cells occurring singly or in pairs. Colonies on bovine blood agar are strongly β -haemolytic, smooth, greyish, non-transparent, shiny and circular, raised, with entire margins, 1-2 mm diameter after 24-48 h at 37°C, and glowing at the periphery having appearance of butyrous consistency [8,9]. All strains are catalase, oxidase, and phosphatase positive, and reduce nitrate [12]. *Gallibacterium* genus can be differentiated from other genera of Pasteurellaceae with catalase, symbiotic growth, hemolysis, urease, indole, acid production from (+) D-xylose, (-) D-mannitol, (-) D-sorbitol, (+) D-mannose, maltose, raffinose, dextrin, ONPG and PNPG tests [18].

Transmission of *G. anatis* infection

The common way of spreading infection is through horizontal dissemination. Infection of month old poultry birds common and appears to be natural [2]. Vertical transmission though not common in Pasteurellaceae family, trans-ovarian infection supporting the vertical transmission has been experimentally proved for *G. anatis* [52]. Isolation of *G. anatis* from the egg yolk and detection of *G. anatis* in a 4-day-old chicken descended from a diseased parent also supported vertical transmission hypothesis for the infection [43,45,53]. Ascending infections appears to be the most probable route for the infection of reproductive organs [38,50]. Venereal transmission of infections also seems to be feasible as the bacterium has been detected

in semen of infected cockerels [54]. Under favourable circumstances, the bacterium can invade systemic circulation from its natural habitats in respiratory and reproductive tract [38,39,55-57].

Virulence factors

RTX toxins are found in many members of Pasteurellaceae and are responsible for the haemolytic and leukotoxic properties in *G. anatis* [58]. *Gallibacterium anatis* biovar haemolytica produces haemolysin like GtxA which is a type of RTX-toxin [60]. GtxA induce a strong leukotoxic effect on avian macrophages and is labelled as a most important virulence factor of *G. anatis* [7,43,59]. GtxA toxin has two domains, C-terminal with homology to other RTX toxin and N-terminal with no homology. C-terminal is responsible for haemolytic function but function of N-terminal is unknown, however, this domain is required for complete haemolytic activity [59]. The four operon genes are responsible for RTX toxin and are transcribed in an order i.e., rtxC, rtxA, rtxB, and rtxD [43]. MARTX (multifunctional autoprocessing RTX) toxins bind and regulate the actin protein of cytoskeleton by which microorganism making a path of immune evasion strategy [60].

Gallibacterium anatis have capability to adhere on the epithelial cells of chicken and other host cells [61,62] by short fimbriae [63]. A number of fimbriae of different sizes and shapes have been defined belonging to the F17-like fimbriae and are grouped in 1-3 different fimbrial cluster [64]. The flfG gene cluster is responsible for adhesion protein of F17 family [65]. One of the detected fimbriae seems to be type IV-like pili having bundle structure formed by thin filament like other pathogenic microorganisms evincing type IV pili [66]. Type IV fimbriae are appendages participating in intercellular motility, microcolony formation, colonization, and the secretion of proteases by host tissues [67].

Gallibacterium anatis produces outer membrane vesicles (OMVs) *in vitro* [65] similar all other Gram-negative bacteria. Virulence properties of OMVs shown by microorganism are adherence, colonization, binding and removal of antibacterial substances along with antibiotics which envisage the survival of microorganism [68,69]. Beside, periplasmic components, compounds of cytoplasmic origin such as DNA have also been found as components contents in OMVs [69,70].

Gallibacterium have capsule in some strain which may contain virulence properties as seen in *Pasteurella multocida* [71]. Capsule is a general structure made up of extracellular polysaccharide and has been reported in both Gram-negative and Gram-positive pathogens [72]. The presence of a thin capsule on *G. anatis* has been observed by transmission electron microscopy [73]. The presence of a capsule in primary culture and disappearance after sub-culture is common finding [14].

Gallibacterium anatis metalloproteases may be having role in infection process [74] are extracellular Zn containing enzymes [74-76], however, their exact role is still to be understood.

Ability of a bacterium to form biofilm indicates its ability of adherence to surfaces and live tissues and plays role in pathogenesis of persistent and chronic infections with increased resistance to antimicrobials [77,78]. Biofilm forming ability varies between isolates of *G. anatis* and categorises as weak, moderate and strong biofilm producer [79].

Some strains of *G. anatis* agglutinate avian erythrocytes [80,81]. The presence of a potential haemagglutinin in OMVs released from *G. anatis* has been observed [79,82]. The activity of haemagglutinating (HAs) to RBC of host are of mainly depend on two type adhesins naming filamentous adhesins and non-filamentous adhesins of pathogens [83]. The type identity of adhesins of *G. anatis* involve in HAs are still unknown [81]. Some of these haemagglutinins could be responsible for the observed agglutinating activity of some strains [79,82-84].

Clustered regularly interspaced short palindromic repeats (CRISPRs) consider as a defence system of bacteria against foreign invasive DNA, such as DNA from phages and plasmids. The difference of natural competence have been explicate between strains of *G. anatis* by CRISPRs interfere in the process of transformation [85,86].

Integrative conjugative elements (ICE) have genes which are present within these elements are capable to excise and integrate in the genome [87]. Identification of ICEs have been reported in the genomes of *G. anatis* [79]. As we know that antimicrobial resistance have been reported in the large number of isolates of *G. anatis* with the possibilities of association to ICEs [19]. Genes encoding fimbrial clusters (flf) have been reported with adjacent mobile elements [64].

Small colony variants (SCVs) have been observed with differences of haemolytic activity [88], in primary cultures of *Gallibacterium* [45,88,89]. Increased persistence, recurrent infections and increased resistance towards antimicrobials have been observed in association with SCVs [8].

Pathogenesis

Repeated isolation of *G. anatis* from the trachea and cloaca of healthy birds indicates its commensal status in the upper respiratory tract and lower genital tract of healthy chickens [2,14,49,50,55,90]. However, isolation of *G. anatis* in association with a wide range of different pathological lesions, including septicaemia, pericarditis, hepatitis, oophoritis, follicle degeneration, enteritis, upper respiratory tract lesions, salpingitis and peritonitis revealed its importance as an opportunistic pathogen [2,17,36,39-43,45,47,55,88,89,91,92]. Recent investigations confirmed that *G. anatis* colonizes the upper respiratory tract without causing clinical signs, whereas it may cause severe lesions in the reproductive tract [54,56]. Studies established *G. anatis* as the most common single bacterial infection in chickens causing reproductive tract disorders [36]. Simultaneous infection with other microorganisms [39,47,48], hormonal influences [42,43], age [2,45], seasonal changes [36], stress [15], cold stress [47], and compromised immunological status [46] are a few predisposing factors nurturing the infection of *G. anatis*. In experimental infections semen quality has been found to be reduced significantly due to decrease in sperm density, total motility with progressive motility, and membrane integrity [54].

Disease associated with *G. anatis* infection

Due to vast range of pathological manifestations of *G. anatis* infection it is difficult to decide the exact disease condition caused by *G. anatis*. Incidences of infection to chicken increase during the peak and late phases of production period [41]. In diseased birds' mortality might take place mainly due to salpingitis, oophoritis and peritonitis. Respiratory tract infections might be responsible for major economic losses due to the rise in treatment cost and losses due to higher condemnation rates and mortality.

Gallibacterium may be causing primary or secondary infections leading to fatal bacteremia, septicaemia and acute septicaemia [41]. The severity of clinical signs, duration of the disease and mortality rate are variable and influenced by environmental factors, such as poor hygiene, inadequate management ventilation, ammonia levels in poultry premises and concurrent diseases. Study on pathogen-specific genes of *Gallibacterium* populations [79] suggested the ability of the pathogen to cause lesions in reproductive organs such as folliculitis, ruptured and haemorrhagic follicles as well as a drop in egg production in adult hens [22,38,55,90]. Haemolytic *G. anatis* was associated with infection in birds kept in alternative husbandry systems and suffering from reproductive disorders [38].

Clinical sign and lesion on *G. anatis* infection

Normally the signs and symptom of diseases caused by *G. anatis* infection in chicken are not pathognomonic leading to creation of confusion between the different similar symptomatic disease like Newcastle, fowl cholera and bird flu. The clinical sign are unspecific but include depression, diarrhoea, pasting around the vent and loss of egg production take place around peak of lay [41]. Mirle et al. [36] examined 496 hens with reproductive tract lesions and isolated *Gallibacterium* in pure culture from 23% of the diseased organs. Even though haemolytic isolates are primarily diseased causing biovar but non-haemolytic strains might be associated with chronic cases of localized or generalized purulent peritonitis with *E. coli* [41].

Diagnosis of *Gallibacterium* infection

Gallibacterium anatis infection can be confirmed only through agent isolation characterised by phenotypic and genotypic methods. The difference between the genomospecies 1 and 2 is possible only through genotypic methods due to the phenotypic heterogeneity among strains [12]. Presently the best way to identify the *Gallibacterium* is its phenotypic (Table 1) characterisation [12] or with GAN850, a *G. anatis* specific probeat position between 850-867 of 16S rRNA [93]. A number of genotypic methods have been established for identification of *Gallibacterium* [12,18,49]. The specificity of these methods, however, remains to be investigated [11]. *Gallibacterium* has a relatively short internal transcribed 16S to 23S rRNA gene sequences compared to other members of Pasteurellaceae, based on the information the *Gallibacterium* specific PCR [18] targeting on 16s rRNA sequence are designed [94]. These Specific ITS-PCR able to amplifies selectively *Gallibacterium* DNA and generating short fragments compared to other members of Pasteurellaceae [95-97]. Primers chosen with specificity for *G. anatis* are 1133fgal (5'-TATTCTTTGTTACCARCGG-3') and 114r (5'-GGTTTCCCCATTTCGG-3') [18,98]. Besides this, AFLP typing method and pulsed-field gel electrophoresis are found to be useful for distinguishing between closely related *G. anatis* clones, thus enabling recognition of specific pathogenic clonal lineages [99-101].

Rapid and specific identification of individual bacterial cells can be achieved by the fluorescent in situ hybridization technique (FISH), based on fluorescent labelled oligonucleotides complementary to bacterial 16S rRNA. This method has advantages over the traditional culture based methods due to its ability to identify live/intact cells as well as non-cultivable organisms [49]. Rapid and accurate identification of related organism can be made along with *G. anatis* in human being with help of gene identified like soda gene [102].

Serological studies also have been found to be helpful in detection of *G. anatis* specific antibodies in infected tested sera by latex agglutination test and enzyme-linked immunosorbent assay (ELISA) [41].

Characteristics	<i>G. anatis</i> biovar haemolytica	<i>G. anatis</i> biovar anatis	<i>G. genomospecies</i> 1	<i>G. genomospecies</i> 2	<i>G. genomospecies</i> 3
B-Haemolysis	+	-	+	+	-
Production of acid from:					
(-) D- Arabinose	(+)	-	(+)	D	D (+/-)
(+) L- Arabinose	-	-	D	D	D (+/-)
Mannitol	+		D	D	
m-Inositol	D	D	-	D	
(-) D- Sorbitol	D	D	-	-	D (+/-)
(-) L- Fucose	(+)	-	+/(+)	D	D (+/-)
Maltose	D	-	+	+	
Trehalose	D	+	+	D	-
Dextrin	D	-	+/(+)	+	-

Table 1: Phenotypic characters of *Gallibacterium* species [12]. Note: Characters are scored as: +, ≥ 90% of strains positive within 1–2 days; (+), ≥ 90% of strains positive within 3-14 days; -, <10% of strains positive within 14 days; d, 11–89% of strains positive, D, doubt (+/-) variation from strain to strain.

Prevention and control of *G. anatis* infection

A pan-genomic reverse vaccinology (RV) approach has been applied to identify novel and potentially broadly protective immunogens from *G. anatis* [84,103-105]. Screening approaches of reverse vaccinology have been applied to identify potential immunogens for 71 proteins in which only one protein contain immunization properties [106]. FlfA and GtxA-N have potential to induce a protective immunity in the homologous strain of *G. anatis* [83]. Although, some commercial vaccine are available for more prevalent three biovar [40] but there is no potential vaccine globally present in the market to control *G. anatis* infection and still protection under field condition remains to be investigated against commercial vaccine [40]. Only antimicrobial chemotherapy is the available method for containment of the disease caused by *G. anatis*, however, it is also proven that *G. anatis* rapidly acquires resistance [40]. Besides, general hygienic measures can be taken in the way similar to control of other contagious diseases in poultry farms.

Antimicrobial drug resistance in *Gallibacterium*

Emergence of antimicrobial resistance has been observed among several organisms belonging to the Pasteurellaceae family [5,106] including *G. anatis* isolates. Though the infection of *Gallibacterium* is treatable with antibiotics, the frequency of treatment failure of *Gallibacterium* seems to be a recurrent problem [107,108]. Resistance in chicken origin isolates of *Gallibacterium* is documented to novobiocin, tylosin, clindamycin, spectinomycin, tetracycline antimicrobials and penicillin [20-26,52,109]. In contrast, Berge et al. reported rarity of tetracycline resistance in *G. anatis* of sheep and goat origin [27].

Conclusions

Gallibacterium anatis especially biovar haemolytica has emerged as an important pathogen of broilers and layers in several countries. However, in lack of elaborate scientific understanding of the pathogen and vaccine(s), efforts to control outbreaks and prevent the disease is a big challenge for poultry scientists and microbiologists.

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