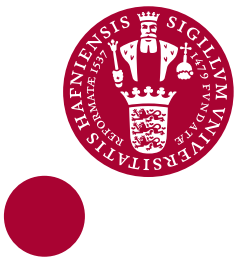

First report of Gallibacterium isolation from layer chickens in Iran

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Short Communication

First report of Gallibacterium isolation from layer chickens in Iran

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ABSTRACT

Gallibacterium, a gram-negative coccobacillus, is a genus of the Pasteurellaceae family with capability of infecting a wide range of avian host species. In 2003, Gallibacterium was selected instead of different previous names. Four different species has been recognized in Gallibacterium genus so far. Members of the genus can cause a wide range of pathological lesions, from upper respiratory tract lesions, follicle degeneration, enteritis, pericarditis, hepatitis, oophoritis, septicemia to more important problems such as salpingitis and peritonitis. Salpingitis and peritonitis of layer farms caused by Gallibacterium result in decreased egg-production and increased mortality. Gallibacterium is the most common single bacterial infection in egg-laying farms with reproductive disorders. During a microbiological survey on bacterial infectious agents of reproductive tract of layers, some isolates from salpingitis cases were suspicious of Gallibacterium. According to culture and biochemical properties, the primary identification was in support of genus Gallibacterium. A polymerase chain reaction (PCR) test using primers specific for the genus identification was carried out. The suspected isolate showed bands of 970 and 1080 bp, which are specific for Gallibcterium. To confirm the identification, a positive sample was sent to University of Copenhagen for repeating the PCR test. To our knowledge, this is the first report of isolation and identification of Gallibacterium in Iran. This study shows the possibility of a role for Gallibacterium in poultry industry of Iran. This study also shows the need for further investigations on epidemiological situation of the infection, as well as the isolation and identification of different species of the genus Gallibacterium.

Keywords: Gallibacterium, Salpinigitis, Chicken, Isolation, Polymerase chain reaction (PCR)

Premier rapport sur l'isolation de Gallibacterium de la couche des poulets en Iran

Résumé: Gallibacterium, un cocobacillus gram-négatif est un genre de la famille des Pasteurellaceae capable d'infecter un large éventail d'espèces hôtes aviaires. En 2003, Gallibacterium a été sélectionné au lieu de noms antérieurs différents. Quatre espèces différentes ont été reconnues dans le genre Gallibacterium jusqu'à présent. Les membres du genre peuvent causer un large éventail de lésions pathologiques, des lésions des voies respiratoires supérieures, la dégénérescence des follicules, l'entérite, la péricardite, l'hépatite, l'oophrite, la septicémie à des problèmes plus importants tels que la salpingite et la péritonite. La salpingite et la péritonite des fermes de couche causées par Gallibacterium entraînent une diminution de la production d'œufs et une mortalité accrue. La gallibactérie est l'infection bactérienne unique la plus fréquente dans les fermes pondeuses atteintes de troubles de la reproduction. Au cours d'une étude microbiologique sur les agents

infectieux bactériens des couches reproductives, certains isolats provenant de cas de salpingite étaient soupçonnés de Gallibacterium. Selon la culture et les propriétés biochimiques, l'identification primaire était à l'appui du genre Gallibacterium. Un test de réaction en chaîne par polymérase (PCR) utilisant des amorces spécifiques à l'identification du genre a été effectué. L'isolat soupçonné a montré des bandes de 970 et 1080 pb, qui sont spécifiques pour Gallibcterium. Pour confirmer l'identification, un échantillon positif a été envoyé à l'Université de Copenhague pour répéter le test de PCR. À notre connaissance, c'est le premier rapport d'isolation et d'identification de Gallibacterium en Iran. Cette étude montre la possibilité d'un rôle pour Gallibacterium dans l'industrie avicole d'Iran. Cette étude montre également la nécessité de nouvelles recherches sur la situation épidémiologique de l'infection, ainsi que sur l'isolation et l'identification de différentes espèces du genre Gallibacterium.

Mots clés: Gallibacterium, Salpinigite, Poulet, Isolation, Réaction en chaîne par polymérase (PCR)

INTRODUCTION

Gallibacterium, a gram-negative, non-motile, encapsulated coccobacillus, is a newly designated genus in the Pasteurellaceae family with the capability of infecting a wide range of avian host species (Christensen et al., 2003). The genus is grouped into seven species of *Gallibacterium anatis*, *G. melopsittaci*, *G. salpingitidis*, and *G. trehalosifermentans* and three genomospecies (Christensen et al., 2003; Bisgaard et al., 2009). The organism was introduced for the first time by Kjos-Hansen in 1950 under the name of "*Cloaca bacterium*" a hemolytic bacterium isolated from acute salpangitis and peritonitis isolated from healthy chicken with no clinical manifestations. Several names, such as *Actinobacillus salpingitidis* and Pasteurella haemolytica-like organisms, were previously used before designation of Gallibacterium, a name adopted by Christensen in 2003. In particular, the haemolytic biovar of *G. anatis* is known to be associated with egg dropping and oophoritis, hepatitis, salpingitis, peritonitis, follicle degeneration, peritonitis septicemia enteritis, and respiratory diseases in chickens (Bojesen et al., 2004; Proctor et al., 2006; Kristensen et al., 2011; Wang et al., 2016). *Gallibacterium anatis* can infect different bird species such as turkeys, geese, ducks, pheasants, partridges, budgerigars, peacock, cage birds, and wild birds, as well as large animals such as cattle and pig (Singh et al., 2016). Although infection of G. anatis may be treatable with antibiotics, some cases of treatment failure and recurrence were reported (Bojesen et al., 2011; Singh et al., 2016). Furthermore, multi-drug resistant strains of G. anatis are resistant to sulpha-based drugs, novobiocin, tylosin, clindamycin, tetracycline, as well as penicillin (Hendriksen et al., 2008; Jones et al., 2013; Singh et al., 2016). Different diagnostic tests were introduced for the identification of Gallibacterium including morphology and biochemical tests, fluorescence in situ hybridization, genus-specific Gallibacterium PCR-based method, MALDI-TOF mass spectrometry, and gtxA gene-based quantitative PCR assay (Alispahic et al., 2011, 2012; Huangfu et al., 2012; Wang et al., 2016). Here, we setup a conventional PCR-based method to detect Gallibacterium genus besides the conventional culture-based techniques. To our knowledge, there is no report of detection of Gallibacterium genus in Iranian flocks of layers and broilers. This article presents the first report of isolation and identification of Gallibacterium from layer chickens in Iran.

MATERIALS AND METHODS

Collection of samples. Samples were taken during a microbiological survey from an industrial poultry abattoir named Zeitoonkar in Mohammadshahr, Karaj, Iran. In so doing, freshly slaughtered egg-laying hens from industrial farms with clinical signs of salpingitis were chosen for necropsy. Swab samples from ovaries, oviduct, uterus, cloaca, and abdominal cavity were inoculated by streak line on to blood agar petri plates with 5% sheep blood (BA) and kept in clean plastic bags in order to transfer them to laboratory for further

steps of isolation and identification of bacterial agents. Then, inoculated plates were incubated overnight at 37 °C. After incubation, the suspected single colonies were sub-cultured on BA according to their appearance and incubated overnight. Sub-cultured isolated single colonies were picked up and suspended in buffer for DNA extraction. In the meantime, a subculture was prepared on brain–heart infusion (BHI) broth for cryo-preservation of the isolates and banking at -80 °C. Identification based on morphology and biochemical tests. For identification of isolated single colonies, beta haemolysis-positive, gram-negative, oxidase-positive, catalase-positive, urease-negative, nitrate-positive, indole-negative, MacConkey positive, glucose-positive, sucrose-positive, and mannitol-positive were carried out. Although in case of Gallibacterium, identification based on morphology and biochemical tests gives a good indication. This should be further confirmed by performing one or more molecular methods such as PCR techniques.

DNA extraction and preparation. Extraction of DNA from the suspected isolates was based on boiling-lysate method according to Wang et al. (2016). In brief, the swabs were soaked in 500 µl of 0.9% saline solution for 10 min and 1 ml of overnight cultured bacteria centrifuged at 14,000 g for 2 min. Then, pellets were re-suspended in 500 µl of 0.9% saline solution, centrifuged for 2 min at 14,000 g, the supernatant was discarded and the pellet was re-suspended in 100 µl of double distilled water (DDW).The achieved suspension was incubated in a heating block at 99 °C for 10 min and centrifuged for 2 min at 14,000 g. Finally, 100 µl of the suspension was stored at -20 °C for further tests, and 2 µl of the supernatant was used for each PCR reaction.

PCR method for detection of Gallibacterium genus. A genus-specific PCR test based on the internal transcribed spacer (ITS) separating 16S and 23S rRNA genes was used as a confirmatory test following cultural isolation of suspicious colonies. Primers used for the conventional Gallibacterium-specific PCR were designed as previously described by (Bojesen et al., 2007). All the materials and primers for PCR assay were prepared by Sinaclon Co., Iran. Genus-specific forward and reverse primers were 1133fgal (5-TATTCTTTGTTACCACGG-3) and 114r (5-GGTTTCCCCATTCGG-3), respectively. The 20 µl PCR reaction was used as follows: 10 µl of Taq PCR Master Mix 2x, final concentration of 0.25 pm/µl for each primer, 3 µl of DDW, and 6 µl of DNA template. The PCR method was carried out under the following program: initial denaturation step for 4 min at 95 °C, followed by 40 cycles of denaturation for 30 s at 95 °C, annealing for 1 min at 55 ℃, extension for 2 min at 72 °C, and a final extension for 10 min at 72 ℃. The PCR products were analyzed using electrophoresis on 1% agarose gel. Also, a 100 bp DNA ladder was loaded beside sample wells (Thermo Fisher Scientific, USA). Gels were stained with ethidium bromide solution. Finally, the stained gel was visualized under UV-transilluminator detector system and photographed. For double confirmation that the genus of positive sample was identified as Gallibacterium, the sample was sent to Department of Veterinary Disease Biology, Faculty of Health Sciences, University of Copenhagen, Denmark. The genus of sample was kindly re-identified by Prof. Anders Miki Bojesen using a PCR assay principally with the same method performed in our laboratory.

RESULTS AND DISCUSSION

Cultural and biochemical tests. As shown in Figure 1A, colonies of *Gallibacterium* are about 1-2 mm grayish, smooth, semitransparent, and slightly raised and circular when incubated for 24 h at 37 °C on nutrient-rich plates containing blood with the capability of hemolysis. Results of biochemical tests on the suspected isolate were gram-negative, hemolysis positive, oxidase, and catalase positive. The isolate was able to grow slowly on MacConkey agar medium in a way that very small colonies were observed after 48 h of incubation at 37 °C. The results of chemical tests for carbohydrates were glucose-, sucrose-, and mannitol-

positive. The tests for urease and indole were negative, while it was positive for nitrate.

PCR tests. The Gallibacterium genus-specific PCR primer set (1133fgal–114r) produced the expected DNA band in all the positive urogenital samples. Figure 2 presents the specific bands of PCR amplicons in gel electrophoresis with approximately 790 and 1080 bp sizes as previously reported by Bojesen et al., 2007. For confirmation of PCR-positive results, as it was described previously, the samples were transferred to Department of Veterinary Diseases Biology University of Copenhagen, for double checking by repeating the PCR tests (Figure 2B). Up to now, Gallibacterium genus has been detected in many European, African, and Asian countries including China, India, Japan, as well as north and south American countries (Singh et al., 2016). *G. anatis* is the most common single bacterial infection in chickens causing reproductive tract disorders. There is no report of isolation and identification of any members of the genus Gallibacterium from Iran. Our report presents the results of a case that presented the infection of an industrial layer chicken's genital tract with Gallibacterium in necropsy at abattoir during a microbiological survey in Karaj, 40 Kilometers west of Tehran, Iran. The survey was followed by laboratory culture-based and chemical characteristics of the suspected isolate for primary identification. The identification was completed at genus level by conducting a PCR test using genus-specific primers, which confirmed the primary identification as Gallibacterium. Since it was for the first time in Iran that Gallibacterium was detected, for further confirmation the isolate was sent to a reference laboratory in Copenhagen, Denmark, where the case was also identified as Gallibacterium. The common route of infection transmission is through horizontal dissemination, though there are few reports on the possibility of vertical transmission (Bojesen et al., 2004; Shapiro et al., 2013; Singh et al., 2016). Ascending transmission is the major route of infection of reproductive organs. Strain of the causative agent, route of transmission, and secondary factors are important epidemiological factors affecting the incidence of the disease (Bojesen et al., 2008). Also, factors related to host (such as hormones, age, stress, and immune system status) and environment (biosecurity level, seasonal changes, and cold stress) are considered as the predisposing factors affecting the growth of the infection in flocks (Persson and Bojesen, 2015; Singh et al., 2016).

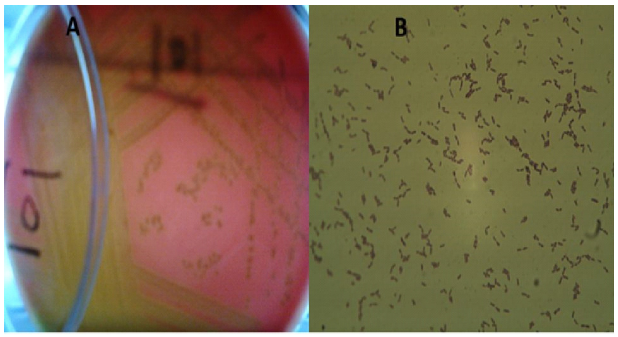

Figure 1. Culture of *Gallibacterium.* (A) A 24-hour culture on blood agar medium. Replace with "Small, smooth, gray and semitransparent colonies with variable sizes of 1-2 mm and a 1-2 mm-hemolysis zone around. (B). A gram-stained smear of a 24-hour culture with the magnification of 1000X. Small red bacteria in pairs and small chains or aggregations are observed.

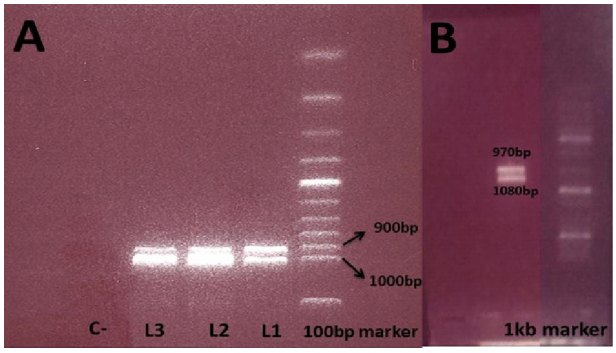

Figure 2. Positive electrophoresis results of polymerase chain reaction from suspected samples of *Gallibacterium* run in Iran (A) and the double-check result in Denmark (B). Bands with 970 and 1080 bp are depicted in figures. In detail of A, lane M is a molecular weight marker (100 bp) and lanes 1 & 4 are specific molecular sized 970 and 1080 bp amplicons representing *Gallibacterium* obtained from Iranian layer chicken flocks. In addition, B, lane M is molecular weight marker (100 bp) and lanes 1 double-checked samples.

From pathological aspect, isolation of *G. anatis* from the trachea and cloaca of healthy birds and a wide range of pathological lesions shows its importance as an opportunistic pathogen (Singh et al., 2016). In industrial poultry production, the importance of infection with different species of Gallibacterium is mainly in layer farms in the form of a sharp reduction around 8-10% at the peak of production, followed by a slow recovery to limits of previous production level (Shapiro et al., 2013). Confirmation of Gallibacterium *anatis* infection can be only achieved by direct isolation of organism and characterization using phenotypic and genotypic methods. The Gallibacterium genus is facultative anaerobe, gram-negative, non-motile, non-spore forming, chemo-organotrophic, and fermentative with rod-shaped or pleomorphic cells. Usually, blood agar colonies are strongly β-haemolytic, smooth, grayish, nontransparent, shiny, circular, raised, with entire margins, and 1-2 mm in diameter after 24-48 h culture at 37°C (Proctor et al., 2006; Bojesen et al., 2008). In addition, all the strains have shown to be catalase, oxidase, and phosphatase positive with the capability of reducing nitrate (Christensen et al., 2003). Moreover, the current tests for differentiation of Gallibacterium genus from other genera of *Pasteurellaceae* are catalase, hemolysis, urease, indole, and capable of acid production from (+) D-xylose, (-) D-mannitol, (-) Dsorbitol, (+) D-mannose, maltose, raffinose, dextrin, ONPG, and PNPG (Bojesen et al., 2007). Previous studies demonstrated that PCR method is 100% specific and 100% sensitive in detection of Gallibacterium genus (Bojesen et al., 2007). Current methods for controlling the diseases caused by *G. anatis* are antimicrobial chemotherapy and general hygiene, although there are noticeable reports on multi-antibiotics drug resistance (Mendoza et al., 2014). Gallibacterium, especially hemolytic isolates, were characterized as an important pathogen of broilers and layers in several countries in the four continents of the world. However, due to lack of knowledge about the pathogenic characteristics and potent vaccine(s), diagnosis, outbreak control, and prevention of disease are a big challenge for both poultry clinicians and microbiologists. To our knowledge, this is the first report of isolation and identification of Gallibactreium in Iran. This study shows the possibility of a role for Gallibacterium in poultry industry of Iran. This study also shows the need for further investigations on epidemiological situation of the infection and the isolation and identification of the different species of the genus Gallibacterium.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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