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R. anatipestifer is a gram-negative, non-motile, non–spore forming, rod-shaped bacterium that infects ducks, geese, turkeys, chickens and other birds, results in contagious septicemia (Hess et al. 2013). Transmission between ducks occurs vertically (through the egg) as well as horizontally via the respiratory tract (Mavromatis et al. 2011). The high economic losses due to infections by this bacterium in duck is due to mortality, with rates ranging from 5% to 75%, and condemnations (Sandhu, 2003). Usually, ducklings of 1 to 8 weeks old are highly susceptible. Birds with chronic form of the disease may develop mucopurulent or caseous salpingitis leading to loss of egg production (Kahn, 2010). Once the disease has invaded duck and goose flocks, it can become endemic and it is difficult to be eradicated (Tsai et al. 2005).

Proper and rapid identification of R. anatipestifer is very essential to avoid the economic losses caused by this bacterium. Beside traditional methods of isolation, other molecular based approaches were developed for its identification. gyrB is a type II DNA topoisomerase, is usually distributed in all strains and can’t spread horizontally among different bacterial species. It is used as a specific marker (Udayan et al. 2019) for identification of R. anatipestifer infection. Although Riemerellosis causes serious economic losses, the pathogenesis of R. anatipestifer and the virulence factors remain mostly unknown and until now.

Some virulence-associated genes such as sspA, hagA1 and prtC were previously identified in R. anatipestifer. sspA, encoding a surface-localized, subtilisin-like serine protease acts in adhesion to fibronectin type III and invasion of epithelial cells (Beckmann et al. 2002; Brown et al. 2005; Cheng et al. 2002). While, hagA1, a gene encoding a serine protease/hemagglutinin, plays an essential role in host colonization by R. anatipestifer (Han et al. 1996). The gene prtC, encodes an extracellular collagenase, was supposed to play an important role in RA-YM’s pathogenicity (a highly virulent field

**Note**

*Molecular Characterization, Virulence and Antimicrobial Susceptibility Testing of Ri Ne Riemerella anatipestifer Isolated from Ducklings*

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This pilot study aimed to characterize *Riemerella anatipestifer* from ducklings, testing their susceptibility to antimicrobial agents and to detect their virulence markers. Seven *R. anatipestifer* isolates with 11.67% infection rate were identified out of sixty freshly dead ducklings and confirmed by PCR assay targeting gyrB gene. The gyrB gene sequences of *R. anatipestifer* isolates were 100% identical to each other and also showed 100% sequence similarity to the published gyrB genes. Four virulence genes namely ompA, prtC, hagA, and sspA were identified in all isolates except sspA was detected in 5 isolates. The antibiogram revealed higher sensitive to imipenem, amikacin, and rifampin, while, a remarkably high resistance was displayed against ampicillin, penicillin, cefipime, trimethoprim/sulfamethoxazole, gentamicin, ceftazidime, streptomycin and cefoperazone. Proper and rapid identification of *R. anatipestifer* with detection of their antimicrobial susceptibility and its virulence potential is essential for understanding the epidemiology of *R. anatipestifer* and to apply the effective control strategies.

**Key words**: Riemerella anatipestifer/ Ducklings / Virulence genes / Antibiogram / phylogenic analysis.

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isolate of *R. anatipestifer* serotype 1) (Wu et al. 2007). Outer membrane protein A (OmpA) is another virulence factor play a very important role in virulence and has been reported to be immunogenic proteins (Hu et al. 2012; Huang et al. 2002).

In spite of the overuse of antibiotics has contributed in the development of drug-resistant strains (Chen et al. 2010; Chen et al. 2012), antibiotics are still used extensively to prevent and control bacterial infection in Egyptian poultry farms. The increasing resistance to the common used antibiotics in *R. anatipestifer* infection extremely challenges the treatment and considered a growing hazard to human health (Chen et al. 2012). Therefore, antibiotics used in the breeding industry require frequent monitoring of the status of the bacterial drug resistance which is very important to guide clinical medication.

There is little data available on *R. anatipestifer* worldwide because of its narrow host range and the scarcity of clinical symptoms in adult birds. The present study aimed to molecular characterization of *R. anatipestifer* strains isolated from ducklings in Egyptian poultry farms, with detection of their virulence potential and their antimicrobial susceptibility.

A total of sixty ducklings from different farms were collected from private laboratories for avian diseases in Mansoura from October 2017 to February 2018. A variable clinical signs were reported including, anorexia, weakness, restlessness, growth retardation, coughing and nasal discharge. Some birds had a history of nervous manifestations as tremors, pyrexia, ataxia and heavy mortality in ducklings of age 4-8 weeks. On necropsy, birds revealed typical perihepatitis, air sacculitis and septicemia. Liver, spleen, lungs, kidneys, heart and nasal swabs of 60 ducklings were transferred to Bacteriology, Mycology and Immunology department, Faculty of Veterinary Medicine, Mansoura University for bacteriological examination. Each sample was individually packed in a clean polyethylene bag and transferred directly to the laboratory in an ice box under aseptic conditions.

Swabs were aseptically taken from the entry of the organs after surface sterilization and directly streaked on 5 % defibrinated sheep blood agar with addition of gentamycin (80 μg/ml) and neomycin-sulphate (25 μg/ml) (Pala et al. 2013), the growing colonies were subsequently streaked on MacConkey’s agar plates. Blood agar was incubated at 37°C for 24–72 hours in an atmosphere enriched with 5 % CO₂ using candle jar with high humidity, while, MacConkey’s agar was incubated for 24–72 hours at 37°C under ordinary conditions. Typical *R. anatipestifer* colonies were grown well on blood agar plates but were usually non-haemolytic and didn’t grow on MacConkey agar. Identification of *R. anatipestifer* based on morphological characteristics and biochemical tests, including, catalase, oxidase, indole production, citrate utilization and urease production. The pure colonies were stored at -20°C in tryptic soy broth containing 20% glycerol for further examination.

Genomic DNA was obtained by boiling (Chang et al. 2019) and a uniplex PCR was performed for further identification of the suspected isolates using *R. anatipestifer* - specific PCR DNA primers targeting gyrB gene. PCR reaction and cyclic conditions were performed following the protocol illustrated by Wang et al. (2012). *ompA* gene was selected for further identification of *R. anatipestifer* isolates and as a one of the most important selected virulence genes using the primer pairs and PCR protocol illustrated by Subramaniam et al. (2000). Three virulence- associated genes (prtC, hagA, and sspA) were selected for molecular characterization according to Han et al. (2013). PCR programme was carried out in a 96 well Applied Biosystem, 2720 thermal cycler. PCR products were electrophoresed in 1.5% agarose gel stained with 0.5 μg/ml ethidium bromide and visualized under UV trans-illumination.

The purification of amplified products of *gyrB* gene for the identified isolates was performed using a Gene JET PCR purification kit (Fermentas, EU) and sent for sequencing to Macro gene Company, South Korea. The resulting DNA sequences were aligned by means of Bloedit software and sequence data were subjected to BLAST search (http://www.blast.ncbi.nlm.nih.gov) to search for similarity and identify of Riemerella at species level. The *gyrB* gene sequences have been deposited at gene bank under the following accession numbers: MK911743, MK911744, MK911745, MK911746, MK911747, MK911748 and MK911749. Phylogenetic tree was constructed using Mega 6 software by Neighbor-joining method (Tamura et al. 2013).

*R. anatipestifer* isolates were assessed by Kirby-Bauer disk-diffusion method for their antibiotic susceptibility on Mueller-Hinton agar plates (Oxoid, UK) supplemented with 5% sheep blood. The bacterial suspension in sterile normal saline was adjusted to the final concentration using a 0.5 McFarland standard and was inoculated into the agar plates, incubated in 5% CO₂ for 18–24 h at 37°C (Agustin et al. 2005). Eleven antibiotic discs (Oxoid, UK) have been selected, including: penicillin G (P;10U), amikacin (AK; 30 μg), ampicillin (AM; 10μg), gentamicin (CN; 10 μg), streptomycin (S; 10μg), trimethoprim/sulphamethoxazole (SXT; 23.75/1.25 μg), rifampin (RA; 5μg), cefoperazone (FEP; 75 μg), cefazidime (CAZ; 30μg), imipenim (IPM; 10 μg) and cefepime (FEP; 30 μg). Multiple drug resistance (MDR) was defined as resistance to at least 3 antimicrobial classes. The multiple antibiotic resistance (MAR) index
was calculated by dividing the total number of resistances to antimicrobials for each isolate by the total number of tested antimicrobials (Krumperman, 1983).

Based on the morphology, cultural characteristics and biochemical reactions of the isolated organism, 11.67% (7/60) of the examined ducklings collected from different farms were tested positive for *R. anatipestifer* infection. These isolates were confirmed by uniplex PCR assay targeting *gyrB* gene. PCR assay could successfully amplify a fragment about 194 bp from all *R. anatipestifer* isolates (Figure 1). Similarly, a prevalence of 11.7% was reported in Egypt from ducks (Deif et al. 2015). While, a higher prevalence (46%; 26/56) was reported in China (Wang et al. 2012) and Korea (39.0%; 48/123) (Wei et al. 2013). This may contributed to that the clinically healthy ducks could still carry *R. anatipestifer*.

Identification of *R. anatipestifer* by traditional methods are often inadequate because of its phenotypic diversity and variable reactions, moreover, available PCR tests up to now are not specific for this species (Christensen and Bisgaard, 2010). Recently, sequence-based approaches are becoming more frequently use due to its ease of use and compare the results via internet as well as reproducibility (Cha et al. 2015). In this study, the sequence analysis showed that *R. anatipestifer* isolates were 100% identical and sequence analysis of *gyrB* gene revealed a 100% similarity between three strains MK911743, MK911744 and MK911745 and the other four strains namely MK911746, MK911747, MK911748, MK911749 also have 100% similarity indicating that all isolates had a common source of infection.

Outer membrane protein A was detected in all *R. anatipestifer* in this study (Figure 2). It is considered one of the most important virulence factor in *R. anatipestifer* and it induces a strong antibody response which responsible for cellular adhesion and invasion (Hu et al. 2012). It could be additionally used as a target to differentiate *R. anatipestifer* from other bacterial species (Subramaniam et al. 2000).While, *prtC* gene with product lengths 839bp (Figure 3) and *hagA* gene with product lengths 565bp (Figure 4) were detected in all isolates, but, *sspA* gene with product lengths 786bp were detected in five isolates of *R. anatipestifer* with a prevalence rate of 71.4% (Figure 5). These results are consistent with other previous study used these three virulence- associated genes in Korea (Han et al 2013) and in china, Zhou et al. (2011).

A phylogenetic tree was generated based on the sequences of *gyrB* gene (Figure 6). The *gyrB* gene sequences of MK911746, MK911747, MK911748 and MK911749 were 100% identical to each other and also showed 100% sequence similarity to the published *gyrB* genes of CP002346, CP003388, CP002562, CP002346.1, CP003388.1, CP002562.1,
CP045564.1, CP003787.1, CP004020.1, CP007503.1, CP007504.1, CP007204.1, LT906475.1 and 97.38% similarity to the known gyrB genes of CP011859.1. While, MK911743.1, MK911744.1, MK911745, MK911746, MK911747, MK911748 and MK911749 were 100% identical to each other, 98.45% to CP006649.1, 97.94% to CP041029.1 and CP029760.1.

In this study, _R. anatipestifer_ isolates displayed significant resistance to most antimicrobial classes used including trimethoprim/ sulfamethoxazole, cefepime, streptomycin, ceftriaxime & ampicillin, penicillin, gentamycin and cefoperazone (Table 1). On the other hand, _R. anatipestifer_ isolates were highly sensitive to amikacin, imipenem and rifampin. These results nearly agreed with Zhong et al. (2009); Chang et al. (2019); and Soman et al. (2014). The multiple drug resistances were found in all _R. anatipestifer_ isolates (Table 2). Moreover, MAR index were calculated, values range from 0 to 1. A value > 0.2 reflects high risk source of contamination due to the previous exposure of the bacterial isolates to numerous antibiotics. In contrary, a value < 0.2 indicates that the isolates were less exposed to antibiotics (Subramani et al. 2012). In this study, all resistance patterns had a MAR value > 0.2 indicating the over use or misuse of these antibiotics in poultry farms.

In summary, this study proposes sequence information for _R. anatipestifer_ which will help as a base of future
studies that will facilitate the understanding of the role of *R. anatipestifer* in the health of ducklings. Detection of virulence associated genes of *R. anatipestifer* helps in understanding virulence mechanisms possess by this bacterium and improving the strategies used for disease control. Finally, a continuous and frequent monitoring of antimicrobial resistant is necessary to determining the most effective antibiotic for the control of *R. anatipestifer* duckling infections.

**Conflict of Interest:** The authors declare no conflict of interest

###TABLE 1. Antimicrobial susceptibility of *R. anatipestifer* to different antimicrobial agents.

| Antimicrobial agent     | Disc Code | Antimicrobial class | R. anatipestifer (n=7) |
|-------------------------|-----------|---------------------|------------------------|
| Penicillin              | P         | Beta - Lactam       | Sensitive: 2 (28.6%)   |
|                         |           |                     | Resist: 5 (71.4%)      |
| Ampicillin              | AM        | Beta - Lactam       | Sensitive: 2 (28.6%)   |
|                         |           |                     | Resist: 5 (71.4%)      |
| Gentamicin              | CN        | Aminoglycosides     | Sensitive: 0 (0.0%)    |
|                         |           |                     | Resist: 1 (100%)       |
| Streptomycin            | S         | Aminoglycosides     | Sensitive: 1 (14.3%)   |
|                         |           |                     | Resist: 6 (85.7%)      |
| Amikacin                | AK        | Aminoglycosides     | Sensitive: 7 (100%)    |
|                         |           |                     | Resist: 0 (0.0%)       |
| Trimethoprim / sulfamethoxazole | SXT   | sulfonamides        | Sensitive: 1 (14.3%)   |
|                         |           |                     | Resist: 6 (85.7%)      |
| Cefoperazone            | FEP       | β-lactams           | Sensitive: 2 (28.6%)   |
|                         |           |                     | Resist: 5 (71.4%)      |
| Ceftazidime             | CAZ       | β-lactams           | Sensitive: 1 (14.3%)   |
|                         |           |                     | Resist: 6 (85.7%)      |
| Cefepime                | FEP       | β-lactams           | Sensitive: 1 (14.3%)   |
|                         |           |                     | Resist: 6 (85.7%)      |
| Imipenem                | IPM       | β-lactams           | Sensitive: 7 (100%)    |
|                         |           |                     | Resist: 0 (0.0%)       |
| Rifampin                | RA        | Rifampin            | Sensitive: 6 (85.7%)   |
|                         |           |                     | Resist: 1 (14.3%)      |

###TABLE 2. Antimicrobial susceptibility profiles and MAR index of *R. anatipestifer* isolates.

| Strain            | Sampling Site | Virulence Genes | Antimicrobial resistant profile | Number of antibiotics | MAR index |
|-------------------|---------------|-----------------|---------------------------------|-----------------------|-----------|
| MK911743          | Nasal discharge | *ompA, prtC, hagA, sspA* | RA, P, SXT, AM, CN, FEP, S, CEP, CAZ | 9                     | 0.82      |
| MK911744          | Nasal discharge | *ompA, prtC, hagA, sspA* | P, AM, CN, SXT, CAZ, FEP, S, CEP | 8                     | 0.73      |
| MK911745          | Nasal discharge | *ompA, prtC, hagA, sspA* | P, AM, CN, CAZ, FEP, S, CEP | 7                     | 0.64      |
| MK911746          | Nasal discharge | *ompA, prtC, hagA, sspA* | SXT, CAZ, CN, FEP, CEP | 5                     | 0.45      |
| MK911747          | Nasal discharge | *ompA, prtC, hagA, sspA* | SXT, S, CN, FEP, CEP, CAZ | 6                     | 0.55      |
| MK911748          | Nasal discharge | *ompA, prtC, hagA* | P, SXT, AM, CN, CAZ, FEP, S | 7                     | 0.64      |
| MK911749          | Nasal discharge | *ompA, prtC, hagA* | P, AM, CN, SXT, S | 5                     | 0.45      |

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