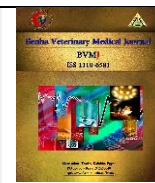




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Unveiling the prevalence and impact of *Riemerella anatipestifer* in Egyptian duck farms: Implications for disease management and vaccine development

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ABSTRACT

Duck septicemia, attributed to *Riemerella anatipestifer*, stands as a significant concern plaguing duck farms globally and specifically in Egypt. The study conducted throughout 2021-2022 aimed to identify and evaluate the incidence of *R. anatipestifer*, the underlying cause of duck septicemia within Egyptian duck farms. A comprehensive analysis was undertaken to furnish invaluable data essential for advancing disease treatment and control strategies. Five hundred ducks exhibiting symptoms akin to septicemia were scrutinized to discern the prevalence of *R. anatipestifer*. Initial assessments revealed that only 35% (175 out of 500) of the bacterial isolates displayed characteristics aligned with *R. anatipestifer*. Subsequent PCR analysis to confirm the presence of *R. anatipestifer* demonstrated that 34% (60 out of 175) of the bacterial strains indeed belonged to this species. These findings underscore the prevalent nature of *R. anatipestifer* among duck populations in Egypt, accentuating the pressing need for robust control measures. Notably, the study meticulously documented the prevalence of *R. anatipestifer* across five Egyptian governorates Monufia, Qalyubia, Gharbia, Sharikya, and El Behera. Understanding the widespread distribution of *R. anatipestifer* in these regions holds paramount importance in the formulation and development of a polyvalent vaccine aimed at mitigating the burden of duck septicemia within Egypt's duck farming industry. This comprehensive analysis provides crucial insights instrumental in devising targeted interventions for managing and containing the prevalence of *R. anatipestifer*, ensuring the sustained health and welfare of the nation's duck population while safeguarding the economic viability of the duck farming sector in Egypt.

1. INTRODUCTION

Riemerella anatipestifer, a Gram-negative, non-motile, non-spore forming, rod-shaped bacterium, has been identified as the causative agent behind septicemia in waterfowl species, predominantly affecting ducks, geese, and turkeys (El-Hamid et al. 2019). Originally described by (Riemer 1904), this bacterium underwent several taxonomic revisions and was previously known by various names, such as *Pfeifferella anatipestifer*, *Moraxella anatipestifer*, and *Pasteurella anatipestifer*, before being reclassified as *Riemerella anatipestifer* by (Segers et al. 1993).

Duck septicemia, attributed to *R. anatipestifer*, is a significant concern in the waterfowl industry in Egypt and globally, resulting in considerable economic losses. Ducks aged between 1 and 8 weeks, especially those under 5 weeks old, exhibit heightened susceptibility to

the disease. Clinical signs are characterized by a range of symptoms, including nasal and ocular discharges, sneezing, incoordination leading to a peculiar lying posture with leg paddling, and a mortality rate varying between 5% and 70%. Postmortem examination often reveals gross lesions such as fibrous polyserositis, air sacculitis, and perihepatitis. Fibrinous meningitis may also develop, indicating involvement of the central nervous system. Chronic manifestations might lead to mucopurulent or caseous salpingitis, significantly reducing egg production. Stressors such as environmental conditions or concurrent infections further exacerbate the susceptibility of ducklings to the disease (Sandhu 2008; Fulton and Rimler 2010; Soman et al. 2014).

R. anatipestifer is a diverse bacterium with at least 21 serotypes identified. Understanding the diverse serotypes is crucial for the development of effective

vaccines and for comprehensive epidemiological investigations (Chikuba et al. 2016). Among the potential vaccine candidates, outer membrane protein A (*ompA*) exhibits high immunogenicity and is considered a valuable diagnostic tool owing to its capacity to detect infections caused by all serotypes. Furthermore, *ompA* holds promise as an antigen in the formulation of future vaccines aimed at preventing infectious septicemia in ducklings (Subramaniam et al. 2000).

In addressing the pressing need for rapid and accurate diagnostics, molecular techniques have gained prominence for detecting *R. anatipestifer*. Polymerase chain reaction (PCR) coupled with sequence analysis of the *ompA* gene has displayed notable sensitivity and specificity, presenting itself as a powerful tool for the swift identification of *R. anatipestifer* infections.

Conducted between 2020 and 2021, this study aimed to investigate the prevalence of distinct *R. anatipestifer* serotypes in duck farms across multiple Egyptian governorates, including Monufia, Qalyubia, Gharbia, Sharikya, and El Behera. The outcomes of this research endeavor aspire to furnish essential insights into the epidemiology of *R. anatipestifer* infections in Egypt,

thereby contributing to the formulation of polyvalent vaccines and more effective strategies for disease control and management.

2. MATERIAL AND METHODS

2.1. Sample Collection

Between November 2020 and December 2021, a comprehensive sampling effort was undertaken across five Egyptian governorates (Monufia, Qalyubia, Gharbia, Sharikya, and El Behera), targeting 500 ducklings and ducks exhibiting symptoms suggestive of bacterial infection. The sampled ducks encompassed diverse breeds, including Muscovy, Pekin, and Mallard. These birds were selected from farms exhibiting prevalent signs of anorexia, depression, and various respiratory indications, such as sneezing, nasal discharge, grasping, and coughing. Notably, the morbidity rates among the examined duck populations ranged from 20% to 60%, with mortality rates estimated between 5% and 50%, primarily affecting ducklings aged 1-4 weeks. A tabulated representation of the collected samples is detailed in Table 1

Table 1 Samples characteristics from the examined survey farms

Governorates	Farm No.	No. of samples	Age/day	Breed	Morbidity %	Mortality %	Season
Monufia	1	35	10	Muscovy	30%	10%	Outum,2020
	2	35	15	Mallard	20%	10%	Outum,2021
	3	35	7	Mallard	40%	5%	Winter,2020
Gharbia	4	35	20	Pekin	40%	15%	Winter,2020
	5	35	15	Muscovy	55%	25%	Winter,2021
	6	35	25	Muscovy	60%	25%	Winter,2021
Sharikya	7	35	30	Pekin	50%	25%	Outum,2021
	8	35	15	Mallard	60%	30%	Winter,2021
	9	35	20	Muscovy	50%	40%	Winter,2021
Qalyubia	10	35	35	Muscovy	60%	30%	Winter,2020
	11	30	8	Muscovy	50%	30%	Winter,2021
	12	30	10	Pekin	40%	50%	Outum,2021
Behara	13	30	10	Mallard	40%	20%	Winter,2020
	14	30	15	Mallard	40%	30%	Winter,2021
	15	30	25	Muscovy	30%	40%	Winter,2021
TOTAL	15	500					

2.2. Bacteriological Isolation and Identification

Samples extracted from heart blood, lung, brain, and liver were aseptically collected and subsequently streaked onto distinct culture media: 10% sheep blood agar (Oxoid), tryptone soya agar (Oxoid), chocolate agar (Oxoid), and MacConkey agar (Oxoid). The cultured plates were then subjected to various incubation conditions, including incubation at 37°C for 48 hours under 5% CO₂ for blood agar and tryptone soya agar, 24-hour aerobic incubation at 37°C for MacConkey agar, and 48-hour incubation at 37°C for chocolate agar. The identification of colonies was meticulously conducted, relying on a comprehensive assessment of morphological attributes, cultural characteristics, and an array of biochemical tests, in alignment with the established protocols outlined by (Pala et al. 2013).

2.3. Pathogenicity Test

To determine the pathogenicity of the isolates, mice aged 6 weeks were employed in rigorous

experimentation. A concentration of 3×10^8 CFU/ml of the 10 isolates was administered intraperitoneally to mice, while ducklings received an identical concentration through intramuscular inoculation. Control groups consisting of two mice and two ducklings were also included in each experimental setup. Subsequent observation and monitoring of these animals extended over 14 days, encompassing the assessment of clinical manifestations and survival rates. Furthermore, post-experimentation efforts were made to reisolate the organism using 10% sheep blood agar from the heart blood, lung, liver, spleen, and brain, following the methodology established by (Soman et al. 2014).

2.4. Molecular Identification via Polymerase Chain Reaction (PCR)

The process of DNA extraction and purification involved tissue homogenates (liver, spleen, and lung) or L.B broth, employing the Qiagen D Neasy Blood and Tissue extraction kit in strict adherence to the manufacturer's guidelines. The purified DNA

specimens were diligently preserved at -20°C in a 100-liter elution buffer. Employing specific primer pairs targeting approximately 608 bp of the *ompA* gene of *R. anatipestifer*, PCR amplification was facilitated using Dream Taq Green PCR Master Mix. The PCR involved a series of steps comprising template DNA preparation, amplification cycles, and a final extension phase, following the established protocol detailed by (Heba et al. 2015). After amplification, gel electrophoresis using a 1% agarose gel was performed, and the resulting products were visualized under ultraviolet transilluminators post-staining with ethidium bromide. The product size was precisely determined by comparison with the Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific). Additionally, PCR was performed with universal primers for *P. multocida*, following cycling conditions adapted from (Townsend et al. 1996), with modifications to include an initial denaturation at 95°C for 5 min and subsequent cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min using a Perkin Elmer Gene Amp PCR system 9700 thermal cyclor.

3. RESULTS

3.1. Bacterial Identification

Upon culturing on various agar plates, specific morphological and staining characteristics of the isolates were identified. Confluent, grey, moist, convex colonies with a dew drop appearance were observed on blood, chocolate, and tryptone soya agar following 48 hours of incubation. However, no growth was observed on MacConkey agar. Gram's staining of bacterial smears from suspected colonies displayed gram-negative, non-spore-forming, non-motile and pleomorphic to short rods shaped with round ends, arranged singly, in pairs, short chains or in filamentous form, by Leishman's-stained blood smears: bipolarity.

3.2. Biochemical Examination

The suspected colonies underwent comprehensive biochemical tests to discern their characteristics further. These tests included motility, growth on MacConkey agar, hemolysis on blood agar, gelatin liquefaction, and several others, as listed in Table 2. The results obtained from the biochemical tests for *R. anatipestifer* identification are presented in tabular format (Table 2).

Table 2 Biochemical identification of *R. anatipestifer*

Test	Result	Test	Result
Oxidase production	+	Galactose fermentation	-
Catalase production	+	Sucrose fermentation	-
Indole production	-	Fructose fermentation	-
Nitrate reduced	-	Mannitol fermentation	-
Urease production	v	Mannose fermentation	-
Methyl red	-	Gelatin liquefaction	+
Voges-Proskauer	-	Motility	-
H ₂ S production	-	Hemolysis on blood agar	-
Glucose fermentation	-	Growth on MacConkey's agar	-

3.3. Incidence of *R. anatipestifer* in Ducks

From the 500 collected ducks exhibiting septicemia symptoms, a subset of 175 (35%) (11 from Monufia which represent 6.2%, 29 from Gharbia which represent 16.5%, 43 from Sharikya which represent 24.5%, 52 which represent 29.8% from Qalyubia, and 40

from Behara which represent 23%) showed initial characteristics of *Riemerella anatipestifer* mainly in the age of 15 to 20 days. Subsequent pathogenicity testing in mice indicated that inoculation with suspected colonies did not induce illness or death. However, experimental infection in ducklings revealed that 4 isolates among 10 suspected bacterial colonies could replicate disease symptoms within 12-48 hours, leading to fatalities. Gross observations exhibited an enlarged and congested liver and spleen, with bipolar organisms observed in Leishman's-stained liver, heart, and blood impression smears. Furthermore, re-isolation on 10% sheep blood agar confirmed the presence of *R. anatipestifer* in the blood, lung, liver, heart, and spleen of diseased ducklings.

3.4. Polymerase Chain Reaction (PCR)

PCR testing of the 175 isolates using specific primers for the 608 bp PCR product of the outer membrane protein *ompA* gene identified 60 out of 175 samples (34%) as *R. anatipestifer*. This confirmed an incidence rate of 12% within the total collected samples. Moreover, the universal primer PCR targeting *P. multocida* yielded negative results, confirming that the observed disease epidemic was not caused by *Pasteurella multocida*. The PCR results are depicted in Fig. 1., showcasing the *ompA* gene among the examined samples, with specific lanes exhibiting positive and negative controls alongside the DNA marker.

4. DISCUSSION

Duck septicemia, caused by *Riemerella anatipestifer* (*R. anatipestifer*), represents a significant threat to the duck industry globally and particularly in Egypt, where it inflicts substantial economic losses (Heba et al. 2015; El-Hamid et al. 2019). This research aimed to comprehensively investigate the prevalence of *R. anatipestifer* in duck farms across five Egyptian governorates—Qalubia, Minofya, Gharbia, Sharkia, and Behara—from November 2020 to December 2021. The emergence of this disease has raised significant concerns, as it not only affects the economic viability of duck farming but also compromises animal welfare. In this study, a substantial incidence rate of 12% was identified, with 60 isolates of *R. anatipestifer* detected from 500 ducklings and ducks exhibiting symptoms suggestive of bacterial infection which matches with the result obtained from (Ibrahim et al., 2005). Bacteriological examination of clinically diseased ducklings revealed 10-12% positive cases, also among the bacteriologically positive 20 birds, only 10 could be identified by PCR as *R. anatipestifer* with a prevalence rate of 8.33% (Abdelrhman et al., 2021).

The observed clinical manifestations in the affected ducks encompassed a range of symptoms, including dizziness, dullness, huddling, poor nutrition, swimming refusal, purulent discharge from the eye and nose, green diarrhea, incoordination, and mortality. These clinical signs are consistent with previous findings, underscoring the severity and complexity of *R.*

anatipestifer infections in ducks with the same incidence rates (Kardos et al. 2007).

Furthermore, examination of the affected ducks revealed gross abnormalities such as meningeal congestion, fibrinous pericarditis, perihepatitis, splenomegaly, and inflammation of air sacs. These pathological findings are in line with documented manifestations associated with *R. anatipestifer* infections, emphasizing the disease's severe impact on duck health (Kardos et al. 2007).

R. anatipestifer is recognized for its diverse serotypes, which pose challenges in accurate diagnosis and hinder cross-protection among different serotypes (Subramaniam et al. 2000; Pathanasophon et al. 2002; Tsai et al. 2005). Studies have reported the presence of at least 21 serotypes of *R. anatipestifer*. Notably, various geographic regions have identified predominant serotypes responsible for disease outbreaks. For instance, major epidemics in China have been linked to serotypes 1, 2, and 10 (Hu et al. 2011), while in Thailand, *R. anatipestifer* serotype 1 has been identified as a major causative agent of disease outbreaks (Pathanasophon et al. 1995).

Interestingly, the current study suggested that the local isolates primarily belonged to serotype 1 and serotype 2, aligning with the prevalent serotypes responsible for the observed epidemic cases within the examined governorates during the specified period. Further detection of the isolates is needed. PCR may serve as a useful molecular tool for subtyping *R. anatipestifer* isolates for epidemiological investigations (Huang et al. 1999).

Detection of *R. anatipestifer* by traditional methods is often not sufficient because of phenotypic diversity, other methods must be used for further accurate and rapid diagnosis; such as phenogenic analysis which would help for rapid confirmation of specific serotypes that cause the disease. The present study confirms that PCR and sequence analysis of *OmpA* gene of *R. anatipestifer* was found to be a highly sensitive and rapid method for the detection of *R. anatipestifer* and could be an alternative method, especially in case of unavailability of reference hyperimmune serum for serotyping of the circulating isolates (Subramaniam et al., 2000).

Effective disease control strategies, including vaccination, are impeded by the lack of specific hyperimmune sera and the presence of multiple serotypes that do not exhibit cross-protection. Consequently, there is a pressing need for rapid and sensitive diagnostic methods to confirm the presence of duck septicemia. Polymerase chain reaction (PCR) technology emerged as a valuable tool in this study. PCR targeting the detection of a 608 bp partial coding sequence of the *ompA* gene of *R. anatipestifer* was utilized due to *ompA* immunogenic properties (Subramaniam et al. 2000).

ompA plays a pivotal role in the virulence of *R. anatipestifer*, influencing the organism's ability to adhere to host cells. Studies have indicated that mutant strains lacking functional *ompA* exhibit reduced virulence and diminished adhesion capabilities to host

cells (Hu et al., 2011). Therefore, leveraging PCR for the detection of *ompA* gene sequences provides a promising avenue for the rapid and accurate identification of *R. anatipestifer*. This approach holds significant potential for disease surveillance, outbreak management, and facilitating early intervention measures.

5. CONCLUSIONS

This study emphasizes the prevalence of *R. anatipestifer* serotypes, predominantly serotype 1 and serotype 2, in causing epidemic forms of duck septicemia within a relatively short period. The application of PCR targeting the *ompA* gene sequence demonstrated its efficacy as a sensitive and specific diagnostic tool. However, to comprehensively address the diverse serotypes and develop effective vaccines, further research endeavors are warranted. These should focus on elucidating additional virulence factors, exploring genetic variations among serotypes and investigating immunological responses to inform the development of serotype-specific vaccines and effective disease management strategies.

CONFLICT OF INTEREST

The authors state that they do not have any competing interests.

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