Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2018 15(4):3598-3605. **OPEN ACCESS**

A trial on preparation of bivalent vaccine against *Riemerella anatipestifer* and *Escherichia coli* in ducks

***Heba M.Soliman[1]; Heba N. Deif [2]; Amal M.Elsawah[1]; Jakeen El-jakee[2]**

[1]Central Laboratory For Evaluation Of Veterinary Biologics (CLEVB), Abbasia, **Cairo.**
[2]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, **Egypt.**

***Correspondence:** hebasoliman98@yahoo.com **Accepted:** 18 Oct.2018 **Published online:** 18 Dec. 2018

Riemerella anatipestifer (R. anatipestifer) is the causative agent of septicemia anserum exsudativa which belongs to the family *Flavobacteriaceae* of Gram-negative bacteria. The goal of the present study was isolation and identification of *R. anatipestifer* from duck septicemia outbreaks in ducklings in Egypt and preparation of a potent bivalent *R. anatipestifer* and *E. coli* vaccine. A total of 190 Muscovy and Baladi breeds ducklings suffered from signs of septicemia were collected to be examined. *R. anatipestifer* isolates were examined, biochemically identified using API 20E system and by PCR based 16S ribosomal RNA. As a trial for disease control, preparation of bivalent vaccine of *R. anatipestifer* and *E. coli* were carried out. Five isolates suspected to be *R. anatipestifer* were detected (2.6%). Preparation of bivalent vaccine of *R. anatipestifer* and *E. coli* was prepared using montanide ISA71 adjuvant. Immune response was assessed using challenge test.

Keywords: Ducklings; *R. anatipestifer*; Duck septicemia; PCR, vaccine.

INTRODUCTION

R. anatipestifer is a Gram-negative bacterium in the family *Flavobacteriaceae* and rRNA superfamily V (Segers et al., 1993). Transmission between ducks occurs vertically (through the egg) as well as horizontally via the respiratory tract (Mavromatis et al., 2011). It can infect ducks, geese, turkeys, chickens, and other birds and leads to a contagious septicemia (Hess et al., 2013).Cha et al., (2015) carried out surveillance for *R. anatipestifer* in wild birds along the East Asian-Australasian flyway in South Korea.

In Egypt, the problem has been persisting, especially in 1-7-week-old ducklings, in areas where duck raising is common since the first report in 1982 (Mahitanan et al., 1982). Once the disease invades duck flocks, it becomes endemic. Eradication is difficult, with repeated possible infectious episodes.

In Muscovy ducks, virus-bacterial associations are often common. Results from different countries show that reovirus and *Escherichia coli* (*E. coli*) are major pathogens, contributing significantly to economic losses to the duck industry, (Kempf et al., 1995).

Although *E.coli* is existing as one of the commensal intestinal flora, where it is responsible for the production of many beneficial metabolic products. It is also capable of causing different disease syndromes in animal and human hosts (Maturana et al., 2011).

Among the respiratory diseases of ducks, *R. anatipestifer* and *E. coli* infections are of primary economic importance to the duck industry. Since the introduction of the *R. anatipestifer* bacterin (Layton and Sandhu, 1984) losses due to *R. anatipestifer* have decreased significantly.

Colibacillosis, however, continues to be a major cause of mortality on many duck farms, in spite of preventive sanitation and management practices. Since both *E. coli* and *R. anatipestifer* infections occur at about the same age and are not distinguishable by clinical signs or lesions (Leibovitz et al., 1972) , it was considered highly desirable to develop and study the efficacy of a combination product for the prevention of both *E. coli* and *R. anatipestifer* infections.

The definitive diagnosis of *R. anatipestifer* infection requires isolation and identification depending on growth, morphological and biochemical characteristics. However, it is characterized more by the absence than by the presence of specific phenotypic properties (Hinz et al., 1998), no selective and/or indicative media have been established for *R. anatipestifer* (Rimler et al., 1998). While serotyping is the traditional method for differentiation between *R. anatipestifer* isolates (Pathanasophon et al., 2002), other methods, including repetitive-sequence polymerase chain reaction (Rep-PCR) (Huang et al., 1999), PCR based on 16S rRNA or *rpoB* genes (Christensen and Bisgaard, 2010), multiplex PCR (Hu et al*.,* 2011), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Rubbenstroth et al., 2013) have also been used to characterize isolates. (Liu et al., 2018) developed genetic methods that provide new and useful tools required to investigate the physiology and pathogenic mechanisms of *R. anatipestifer.* Disease severity of *R. anatipestifer* infection depends on several factors including route of infection, host species, and pathogenicity of the serotype. Virulent serotypes can cause great economic loss to the agricultural community.

Efforts to immunize birds against *R. anatipestifer* are complicated by the occurrence of many serotypes which don't cross protect (Sandhu, 1979). Vaccines, such as bacterin, have been developed to prevent and control infectious serositis in poultry flocks. *R. anatipestifer* bacterin does not always induce cross-protection. Inactivated bacterins have been successfully used for prophylactic immunization but at least two injections are required. Several attempts have been made to immunize ducks against *R. anatipestifer* infection, using either inactivated bacterin or live vaccines. Most vaccines required more than one injection (Layton and Sandhu, 1984). The present investigation aimed to prepare a potent combined inactivated vaccine of *R. anatipestifer* and *E. coli*.

MATERIALS AND METHODS

Bacterial Strains:

R. anatipestifer:

A total of 190 samples were collected from diseased and freshly dead ducklings of 5-21 days of age from different governorates in Egypt as shown in Table 1. Specimens were taken mainly from heart blood, livers and lungs.The collected diseased ducklings showed severe clinical manifestation as exhaustion, drowsiness, loss of appetite, ruffled feathers, swimming in circles, lameness, labored breathing, cyanosis, nervous manifestation, watery green-yellowish diarrhea, respiratory sign (ocular and nasal discharge) and mucous discharge from the mouth.

Escherichia coli:

E. coli O78 was kindly obtained from Central Laboratory for Evaluation Veterinary Biologics (CLEVB), Abbasia, Cairo and was inoculated onto MacConkey agar medium at 37°C for 24 hr. It was confirmed biochemically by using API 20E identification system following the procedures of kit manual and serotyped in AHRI (Animal Health Research Institute, Dokki).

Bacteriological identification of *R. Anatipestifer* :

The collected samples were subjected to bacteriological examination (Brogden et al., 1982) and direct inoculated into trypticase soya broth then incubated at 37˚C for 24 hours. The cultures were inoculated on blood agar and MacConkey agar medium (Oxoid) then incubated at 37˚C for 24-48 hours. The colonies that failed to grow on MacConkey agar medium and non-hemolytic on blood agar with typical morphological appearance were picked up for identification. Suspected colonies were picked up and stained by Gram's stain then examined microscopically for detection of Gram negative microorganism.

Direct smears from heart blood, livers, lungs, spleen were prepared and stained by Leishman's stain for demonstration of the bipolarity of *R. anatipestifer* under microscope. Pure culture of the suspected isolates was identified biochemically using API 20E identification system (bioMérieux).

Table 1: number and source of the examined samples.

Source of samples	Diseased ducklings	Freshly dead ducklings	Total
Muscovy	92	42	134
Baladi	30	26	56
Total	122	68	190

Molecular identification of *R. anatipestifer* isolates by16s rRNA PCR:

DNA was extracted from the isolates using QIAMP DNA Mini Kit (according to instruction of the manufacture) and was subjected to a previously standardized PCR assay according to (Tsai et al., 2005).

Primer pairs (Biomatik) [the sense primer (RA20F2: 5'- CAGCTTAACTGTAGAACTGC -3') and the antisense (RA20R4: 5'- TCGAGATTTGCATCACTTCG -3') primer] to detect 16s rRNA gene with expected fragment at 662 bp were used.

Vaccine preparation: (Layton & Sandhu, 1984)

E. coli bacterin was made with cultures grown in tryptose broth (Difco). After 18-24 hr of incubation at 37˚C, purity was checked on trypticase soy agar and Eosin Methylene Blue agar plates (Difco). Viable colony forming units (CFU) were determined by the serial dilution method. Formalin was added to a final concentration of 0.4% for inactivation. *R. anatipestifer* bacterin was prepared as described in *E. coli* bacterin preparation. Then Montanide ISA71 (SEPPIC®, France) was mixed with one part of bacterial suspension in a ratio of 71 adjuvant: 29 antigen. Based on bacterial counts, appropriate quantities of each bacterin were mixed to obtain the combinated *E. coli* - *R. anatipestifer* bacterin that contained approximately 10^8 cells of *E. coli* and 10^9 cells of *R. anatipestifer* /dose (0.5 ml).

Immunization. Ducklings were injected subcutaneously (s/c) in the neck with 0.5 ml of the prepared bacterin. Inoculations were given at 2 and 3 weeks of age.

Pekin Ducks:

One hundred: one day-old apparently healthy Pekin ducklings obtained from commercial farm at El-Minufiya Governorate were used for this study. The ducklings had no symptoms for duck septicemia infection and free from *R. anatipestifer*. The ducks were kept and reared in separate pens at the animal husbandry facilities of Central Laboratory for Evaluation Veterinary Biologics (CLEVB) until used for inactivated bacterin experiment.

Experimental design(Layton & Sandhu, 1984):

One hundred ducklings aged 1 week old were divided into:

Group 1:

20 duckling vaccinated s/c with 0.5 ml of the montanide (ISA 71) adjuvanted *R. anatipestifer* inactivated vaccine.

Group 2:

20 duckling vaccinated s/c with 0.5 ml of the montanide (ISA 71) adjuvanted *E. coli* inactivated vaccine.

Group 3:

40 vaccinated s/c with 0.5 ml of the montanide (ISA 71) adjuvanted *E. coli- R. anatipestifer* inactivated vaccine.

Group 4:

10 unvaccinated ducklings (control group) challenged with *R. anatipestifer.*

Group 5:

10 unvaccinated ducklings (control group) challenged with *E. coli.*

Blood samples were collected directly before vaccination and weekly for 3 weeks after immunization and up to 3 weeks after challenge. The collected blood samples were allowed to clot overnight at 4ºC then centrifuged at 3000 xg for 10 minutes. Then all separated sera were stored at -20˚C till used.

Quality control testing of the prepared experimental vaccines (OIE,2013):

Sterility test

The prepared vaccines were tested to be free from any contaminant (aerobic and anaerobic bacteria, fungus and mycoplasma.

Safety test

Safety of the prepared vaccine was tested (OIE, 2013); in which twenty ducklings; 7 days old for each vaccine (10 duckling/ vaccine) were

injected subcutaneously with double doses of the prepared vaccines. Ducklings were observed for 14 successive days to detect any signs of local reaction, clinical symptoms or death.

Challenge test

Ducklings were challenged S/C in the leg 3 weeks after the last bacterin inoculation. The *E. coli* challenge contained approximately 10^8 CFU in 0.5 ml, and the *R. anatipestifer* challenge contained approximately 10^9 CFU/0.2 ml. Mortality was recorded daily for 7 days, after which surviving ducklings were killed and examined for pathological lesions. Selected tissues from dead ducklings were cultured for bacterial isolation and identification.

RESULTS

Isolation and identification of *R. anatipestifer*:

Postmortem lesions included hemorrhages in the heart, liver, gizzard and intestine and white spots and necrotic foci on the liver and spleen. All blood films prepared from blood smears of the infected samples showed bipolar coccobacilli in between RBCs when stained by Leishman's stain.

The produced colonies on 5% defibrinated blood agar were small, convex, transparent, and circular, dew drops like, mucoid, butyrous and by aging they were large and viscous. All isolates could not grow on MacConkey agar or produce hemolysis in blood agar. Smears from colonies; prepared and stained with Gram stain; showed Gram negative rod shape or short coccobacilli organism. Direct smears stained by Leishman's stain showed typical bipolar coccobacilli in-between the RBCs. The isolates were confirmed biochemically using API 20E sysمخtem.

PCR for detection of 16srRNA gene:

4 out of 5 isolates were proved to be positive *R. anatipestifer* using PCR and showed the specific expected PCR products at 662 bps as shown in Figure (1).

Results of quality control of the prepared vaccines:

The prepared vaccines were examined for:

Purity test:

The prepared vaccines were pure.

Sterility test:

The prepared vaccines were free from bacterial and fungal contamination.

Safety test:

The vaccines were found to be safe and no clinical symptoms were appeared when inoculated s/c into ducklings with double dose of the prepared vaccines.

Challenge test:

The data illustrated in Table (2) showed that the protection % against the challenge with *R. anatipestifer* was 90% for G1 and 100% for G3 in comparison with 0% for G4.

The data illustrated in Table (3) revealed that the protection % against the challenge with *E. coli* (O78) was 85% for G2 and 95% for G3 in comparison with 20% for G5.

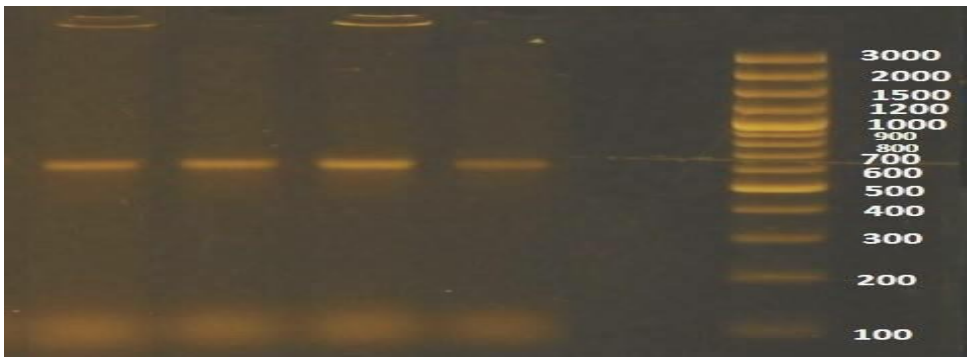

Figure 1: The amplified PCR product (662 bp) specific to *R. anatipestifer*.
Lane1: marker (100bp-3000bp DNA ladder with stain), Lane 2: negative control, Lane 3: positive control (*R. anatipestifer* ATCC# 11845), Lanes 4, 5, and 6: positive isolates.

Table 2. Results of challenge test against *R. anatipestifer* in ducklings vaccinated with combined *R. anatipestifer* and *E. coli* (O78) and *R. anatipestifer* vaccines:

Groups	Total No. of challenged ducks	No. of dead ducks	survived	Protection rate
Group (1)	20	2	18	90%
Group (3)	20	0	20	100%
Group (4)	10	10	0	0

Group 1: duckling vaccinated with montanide ISA 71 adjuvanted *R. anatipestifer* inactivated vaccine
Group 3: duckling vaccinated with montanide ISA 71 adjuvanted *R. anatipestifer- E. coli* (O78) inactivated vaccine
Group 4: unvaccinated ducklings (control group) challenged with *R. anatipestifer*

Table 3. Results of challenge test against *E. coli* (O78) in ducklings vaccinated with combined *R. anatipestifer* and *E. coli* (O78) and *E. coli* (O78) vaccines:

Groups	Total No. of challenged ducks	No. of dead ducks	survived	Protection rate
Group (2)	20	3	17	85%
Group (3)	20	1	19	95%
Group (5)	10	8	2	20%

Group 2: duckling vaccinated with montanide ISA 71 adjuvanted *E. coli* (O78) inactivated vaccine.
Group 3: duckling vaccinated with montanide ISA 71 adjuvanted *R. anatipestifer- E. coli* (O78) inactivated vaccine.
Group 5: unvaccinated ducklings (control group) challenged with *E. coli* (O78).

DISCUSSION

R. anatipestifer infection is a contagious disease of domestic ducks, geese, turkeys and various other domestic and wild birds (Saif, 2008). The disease started in 7-10 weeks old ducks with about 10% mortality. Later spread to younger ducklings of about 3 weeks of age (Saif, 2008).

The present study supports the previous findings of Dudzic (2011) and (Zhou et al., 2011) who recorded that *R. anatipestifer* is a Gram negative, capsulated, bipolar, non-motile, non-spore forming and facultative anaerobic bacteria. The isolates were proven to form small non-hemolytic, dew drop-like non-mucoid, iridescent, smooth, and non-pigmented colonies. All local isolates were subjected to biochemical identification through API 20E system and molecular characterization through PCR. The biochemical reactions of *R. anatipestifer* revealed that all the isolates were indole negative and ornithine decarboxylase (ODC) negative. This finding agreed with the results obtained from (Brenner et al., 2005). Our isolates revealed gelatin liquefaction positive and this is meeting with (Ryll et al., 2001) who reported positive reaction for all tested isolates. Our results showed that *R. anatipestifer* did not grow on MacConkey's agar and have no hemolysis on blood agar. These are in agreement with (Bangun et al., 1981) and Brogden (1989). *R. anatipestifer* infection is always confused with *E. coli* infection, so, early and accurate diagnosis of this infection is important to avoid high loss by mortality. PCR is a highly and specific sensitive and rapid assay for identification of microbial infections, So, this was used in the present study. PCR assay applied on the sequence of 16S rRNA gene which considered a stable sequence on all prokaryotes chromosome. The 16S rRNA based PCR has been employed to detect *R. anatipestifer* (Tsai et al., 2005; Soman, 2014).

In the present study, the 5 positive *R. anatipestifer* isolates showed positive results with biochemical tests undergo PCR for confirmation of biochemical results using 16s rRNA primer showing 4 of 5 positive isolates (80%). Four isolates amplified PCR products at 662 bps specific to *R. anatipestifer.* This result is going in parallel with what was recorded by (Tsai et al., 2005).

Several attempts have been made to immunize ducklings against *R. anatipestifer* infection using inactivated bacterins and live or cell-free culture filtrate vaccines (Zhai et al., 2012). Inactivated bacterins and live vaccines have successfully conferred protection against homologous strains or serotypes of *R. anatipestifer* but were unable to protect against heterologous serotype exposure (Crasta et al., 2002).Among the different trials in duck immunization against *R. anatipestifer* infection, the autogenously monovalent or polyvalent prepared bacterin from local pathogenic strains provided the highest protection against homologous strains under experimental and field condition (Tripathy et al., 1980). Also, Bisgaard (1982) recommended the use of autogenous vaccine for a continuous surveillance to particular serotypes prevalent on each farm (Liu et al.,

2013) developed a trivalent inactivated vaccine of *R. anatipestifer*, including strains CH3 (serotype 1), NJ3 (serotype 2), and HXb2 (serotype 10). (Kang et al., 2018) developed monovalent and bivalent *R. anatipestifer* vaccines that were safe in ducks and provided significant protective efficacy against virulent homologous *R. anatipestifer* strains. *R. anatipestifer* strains are responsible for most of the major disease outbreaks. Therefore, the immunogenicity induced by a broth culture bacterin prepared from local strains was studied in ducks and the protective rates of the prepared autogenous vaccine were investigated. Among the respiratory diseases of ducks, *R. anatipestifer* and *E. coli* infections are of primary economic importance to the duck industry. Since both *E. coli* and *R. anatipestifer* infections occur at about the same age and are not distinguishable by clinical signs or lesions, it considered highly desirable to develop and study the efficacy of a combination product for the prevention of both *E. coli* and *R. anatipestifer* infections. So, in the present study bivalent *E. coli* and *R. anatipestifer* formalin inactivated vaccines were prepared, and the immune response was monitored using challenge test.

The data of the protection percentages in ducklings after challenge with *R. anatipestifer* and *E. coli* reveals that the locally prepared bivalent inactivated *E. coli* and *R. anatipestifer* vaccine induced a considerable immunity in ducklings as it gave early, high and long duration of antibody response. Also, it was efficient and safe in protection of ducklings against *E. coli* and *R. anatipestifer* infections. Depending on the obtained results, it could be suggested to use this bivalent vaccine for control of *E. coli* and *R. anatipestifer* in duck industry.

CONCLUSION

R. anatipestifer is important in veterinary medicine as it is distributed worldwide and causes serious problems in commercial duck and ducklings' flocks.

The results demonstrate the efficacy of inactivated vaccines in preventing *R. anatipestifer* and *E. coli* infections in ducklings; so, this vaccine can be used for its immunization consequently, protection of the duckling's flocks against the hazardous effect of *R. anatipestifer*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors would like to thank central laboratory for evaluation of veterinary biologics (CLEVB), Cairo, Egypt for covering all the expenses of the experiment needed to conduct the work.

AUTHOR CONTRIBUTIONS

HMS performed the experiments and wrote the manuscript. JEL-J, HND and AME designed the experiments and reviewed the manuscript. HMS designed the experiments and prepared the vaccine. All authors read and approved the final version.

REFERENCES

Bangun A,Tripathy DN, and Hanson LE, 1981. Studies of *Pasteurella anatipestifer*: an approach to its classification. Avian Diseases, 25: 326-337.

Bisgaard M, 1982. Antigenic studies on *Pasteurella anatipestifer*, species incertae sedis, using slide and tube agglutination. Avian Pathology, 11: 341-350.

Brenner DJ, Krieg NR, and Staley J, 2005. Bergey's Manual of Systemic Bacteriology. Vol. 2 George M. Garvity.

Brogden KA, Rhoades KR. and Rimler, RB, 1982. serologic types and physiologic characteristics of 46 avian *Pasteurella anatipestifer* cultures. Avian disease, 26 (4): 891-896.

Brogden KA, 1989. *Pasteurella anatipestifer* infection. In: Adlam, C. and L. M. Rutter (Eds), *Pasteurella* and pasteurellosis, pp. 115-129. Academic Press Inc., London, UK.

Cha SY, HS Seo, B Wei, M Kang, JH Roh, RH Yoon, JH Kim and HK Jang, 2015. Surveillance and characterization of *Riemerella anatipestifer* from wild birds in South Korea. J. Wild Dis., 2: 341-347.

Christensen H, and Bisgaard M, 2010. Phylogenetic relationships of

Riemerella anatipestifer serovars and related taxa and an evaluation of specific PCR tests reported for *R. anatipestifer*.*J ApplMicrobiol ,* 108(5):1612-1619.

Crasta KC, Chua KL, Subramaniam S, Frey J, Loh H, and Tan HM, 2002. Identification and Characterization of CAMP Cohemolysin as a Potential Virulence Factor of *Riemerella anatipestifer*. J. Bacteriol, 184: 1932-1939.

Dudzic A, 2011. Avian riemerellosis. [Polish] ZycieWeterynaryjne, 86 (6): 443 - 445.

Hess C, Enichlmayr H, Jandreski-Cvetkovic D, Liebhart D, Bilic I, and Hess M, 2013. *Riemerella anatipestifer* outbreaks in commercial goose flocks and identification of isolates by MALDI-TOF mass spectrometry.*Avian Pathol ,* 42(2):151-156.

Hinz KH, Ryll M, Kohler B, and Glunder G, 1998. Phenotypic characteristics of *Riemerella anatipestifer* and similar micro-organisms from various hosts. Avian Pathology, 27: 33-42.

Huang B, Subramaniam S, Chua KL, Kwang J, Loh H, Frey J, and Tan HM, 1999. Molecular fingerprinting of *Riemerella anatipestifer* by repetitive sequence PCR. *Vet Microbiol,* 67(3):213-219.

Hu Q, Han X, Zhou X, Ding C, Zhu Y, and Yu S, 2011. OmpA is a virulence factor of *Riemerella anatipestifer*. Veterinary Microbiology, 150 (3/4):278-283.

Kang M, Seo HS, Soh SH, Jang HK, 2018. Immunogenicity and safety of a live *Riemerella anatipestifer* vaccine and the contribution of *IgA* to protective efficacy in Pekin ducks. *Vet Microbiol*, 222:132-138.

Kempf I, Gesbert F, Guittet M, Froyman R, Delaporte J, and Bermejean G, 1995. Dose titration study of enrofloxacin (Baytril®) against respiratory colibacillosis in Muscovy ducks. Avian Diseases, 39, 480-488.

Layton HW, & Sandhu TS, 1984. Protection of ducklings with a broth-grown *Pasteurella anatipestifer* bacterin. Avian Diseases, 28: 718-726.

Leibovitz LA. 1972. survey of the so-called "anatipestifer syndrome." Avian Dis. 16:836-851.

Liu H, Wang X, Ding C, Han X, Cheng A, Wang S, Yu S, 2013. Development and evaluation of trivalent *Riemerella anatipestifer*-inactivated vaccine. Clin Vaccine Immunol. 20(5):691-7.

Liu M, Huang Y, Liu J, Biville F, Zhu D, Wang M, Jia R, Chen S, Zhao X, Yang Q, Wu Y, Zhang S, Chen X ,Liu Y, Zhang L, You Y, Yu Y, Cheng A, 2018. Multiple genetic tools for editing the genome of *Riemerella anatipestifer* using a counter selectable marker. Appl Microbiol Biotechnol. 102(17):7475-7488.

Mahitanan W, Bunyanurak W, Suksataichana P, and Arunsakul O, 1982. Infectious serositis in ducklings. Kasetsart Vet, 3:124 - 130.

Maturana VG, Carlos C, Misstretta PM, Amabile C, Nakazato G, Guedes E, Logue CM, and Nolan LK, 2011. Subpathotypes of avian pathogenic *E. Coli* (APEC) exist as defined by their syndromes and virulence traits. J. Microbial., 5(11):55-64.

Mavromatis K, Lu M, Misra M, Lapidus A, Nolan, M.; Lucas, S.; Hammon, N.; Deshpande, S.; Cheng JF, Tapia R, Han C, Goodwin L, Pitluck S, Liolios K, Pagani L, Ivanova N, Mikhailova N, Pati A, Chen A, Palaniappan K, Land M, Hauser L, Jefffries CD, Detter JC, Brambilla EM, Rohde M, Goker M, Gronow S and Woyke T, 2011. Infectious serositis in ducklings. Kasetsart Vet., 3:124 - 130.

OIE TERRESTRIAL MANUAL, 2013. Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.

Pathanasophon P, Phuektes P, Tanticharoenyos T, Narongsak W and Sawada T, 2002. A potential new serotype of *Riemerella anatipestifer* isolated from ducks in Thailand. Avian Pathol, 31(3):267-270.

Rimler RB, Sandhu TS and Glisson JR, 1998. *Riemerella anatipestifer* infection. In D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E. Pearson and W.M. Reed (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn (pp. 22 /23). Kennett Square, PA: The American Association of Avian Pathologists.

Rubbenstroth D, Ryll M, Hotzel H, Christensen H, Knobloch JK, Rautenschlein S and Bisgaard M, 2013. Description of *Riemerella columbi pharyngis sp. nov.,* isolated from the pharynx of healthy domestic pigeons (Columba livia f. domestica), and emended descriptions of the genus *Riemerella*, *Riemerella anatipestifer* and *Riemerella columbina*.*Int J SystEvolMicrobiol* , 63(Pt 1):280-287.

Ryll M, Christensen H, Bisgaard M, Christensen JP, Hinz KH, and Koehler B, 2001. Studies on the prevalence of *Riemerella anatipestifer* in the Upper Respiratory Tract of Clinically

Healthy Ducklings and Characterization of Untypable Strains. J. Vet. Med, B 48: 537-546.

Saif YM, 2008. Diseases of Poultry. 12th Ed. (*Riemerella anatipestifer* Infection), 758-764.

Sandhu TS, 1979. Immunization of White Pekin ducklings against *Pasteurella anatipestifer* infection. Avian Diseases, 23, 662-669.

Segers P, Mannheim W, Vancanneyt M, De Brandt K, Hinz KH, Kersters K and Vandamme P, 1993. *Riemerella anatipestifer* gen. nov., comb. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the Flavobacterium-Cytophagar RNA homology group.*Int J Syst Bacteriol,* 43(4):768-776.

Soman M, Nair SR, Mini M, Mani BK, and Joseph S, 2014. Isolation and polymerase chain reaction-based identication of *Riemerella anatipestifer* from duck in Kerala, India, Vet. World . 7: 765-769.

Tripathy DN, Bangun A, and Hanson LE, 1980. studies of *Pasteurella anatipestifer* in ducks. 2nd. International symposium of veterinary laboratory diagnosticians, Lucerne, Switzerland: 115- 117.

Tsai HJ, Liu YT, Tseng CS and Pan MJ, 2005. Genetic variation of the *ompA* and 16S rRNA genes of *Riemerella anatipestifer*.*Avian Pathol*, 34(1):55-64.

Zhai Z, Cheng L, Tang F, Lu Y, Shao J, Liu G, Bao Y, Chen M, Shang K, Fan H, Yao H, Lu C, Zhang W, 2012. immunoproteomic identification of 11 novel immunoreactive proteins of *Riemerella anatipestifer* serotype 2. FEMS Immunology & Medical Microbiology, 65 (1): 84-95.

Zhou Z, Peng X, Xiao Y, Wang X, Guo Z, Zhu L, Liu M, Jin H, Bi D, Li Z and Sun M, 2011. Genome sequence of poultry pathogen *Riemerella anatipestifer* strain RA-YM (Chinese). J Bacteriol, 193 (5):1284 -1285.