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Research Article

Utility of Molecular Biology Tools for Identification and Characterization of Egyptian *Riemerella anatipestifer* Duck Isolates

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ABSTRACT

Riemerella anatipestifer infection in ducks is an epizootic disease causing huge economic losses. In the current study, samples from 50 duck (five ducks / farm) showing septicemia and polyserositis were collected from four governorates and subjected to phenotypic and genotypic identification to exclude *Pasteurella multocida* due to similar clinical picture. Only 20% (10 out of 50) bacterial isolates gave presumptive characters as *Riemerella anatipestifer* which confirmed by pathogenicity test and genotyping where only four out of 10 samples were PCR positive (40%). The PCR positive samples were subjected to sequence analysis of *OmpA* gene which revealed 100% homology and identity of the four positive samples which revealed that the cause of the epidemic picture in the four different locations in the nearby governorates may be due to the serotype 1 of *R. anatipestifer* which has been verified by both nucleotide and amino acid sequences. Multiple alignment revealed single nucleotide substitution at the base pair number 500 from the start codon of the open reading frame of *OmpA* which lead to single amino acid Substitution from ACA (Threonine(Thr/T)) which is considered as a polar Amino Acid to ATA (Isoleucine (ILe/I) which is a non polar amino acid which is very characteristic for the local isolate that caused the epidemic picture than the rest of isolate and strains. The sequence of this isolate was submitted to Genbank Accession No# MK587441 as GERD/VSVRI/Giza 2018. So, the present study assures that PCR and sequence analysis of *OmpA* gene of *R. anatipestifer* was found to be a highly sensitive and rapid method for detection of *R. anatipestifer* and could be an alternative method especially in case of unavailability of reference hyper immune serum for serotyping of the circulating isolates here in Egypt as the outer membrane proteins (Omp) is a good candidate for serotyping of the organism by sequence analysis.

Key words: *Riemerella anatipestifer*, Ducks, PCR, Egypt

INTRODUCTION

Riemerella anatipestifer is a worldwide pathogen that causing epizootic infection in domestic ducks, turkeys, chickens, pheasants, and waterfowl. In ducks, the infection has many synonyms as new duck disease, duck septicemia, anatipestifer syndrome, anatipestifer septicemia, and infectious serositis. Economic loss to the duck industry from this disease is due to mortality rates ranging from 5- 75%, as well as weight loss and increase rate of condemnations. Ducklings age (1-8weeks old) are highly susceptible especially under 5-weeks old usually die after 1 - 2 days after onset of clinical signs appear; older birds may survive longer. Stress factors such as concomitant disease or adverse environmental conditions, predispose ducklings to outbreaks of the disease (Sandu *et al*., 1991, Fulton and Rimler, 2010 and Soman *et al*., 2014)

Riemerella anatipestifer (*R. anatipestifer*) is a Gram-negative rod-shaped, non-motile, non-sporulating bacterium of the family of Flavobacteriaceae. It was first described by (Riemer, 1904) and then classified as *Pfeifferella anatipestifer*, *Moraxella anatipestifer* and *Pasteurella anatipestifer* (Segers *et al*., 1993).

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There are at least 21 serotypes of *R. anatipestifer* can be useful for epidemiological analysis and a vaccination strategy (Chikuba *et al*., 2016). The high immunoreactivity of outer membrane protein OmpA makes it an interesting candidate for development of specific serological diagnostic tools to detect *R. anatipestifer* infections of all serotypes. In addition, it might be considered as an antigen for designing new vaccines against contagious septicemia of duckling (Subramaniam *et al*., 2000).

Characterization of *R. anatipestifer* by traditional methods is often not sufficient because of phenotypic diversity. So, other methods should be used for further accurate and rapid diagnosis; such as phenogenic analysis which would help for rapid confirmation of specific serotype that cause the disease at the time of an outbreak. The present study suggests that the PCR assay can facilitate fast and proper identification of *R. anatipestifer* infection in ducks. The assay can also differentiate between *R. anatipestifer* and *Pasteurella multocida* and can replace the traditional methods of differentiation which are cumbersome and time-consuming.

MATERIALS AND METHODS

Samples

Fifty samples (5ducks/ farm) of suspected freshly dead ducks were collected from 10 duck farms from different localities (Qalubia, Beni-Sueif, Fayoum and Giza) in Egypt between 2017 and 2018. The examined duck breeds were Pekin, Muscovy and Mallard. The farms were suffered from depression, anorexia and respiratory signs (nasal discharge; sneezing; coughing, and grasping) morbidity rate was ranged from 35-60%, and mortality rate was 8-20% especially at duckling (1-4 weeks old). The history of examined farms was illustrated in (Table 1).

Bacteriological isolation and identification

Samples from heart blood, loopful from lung, and liver were taken and streaked onto 10% sheep blood agar; chocolate agar, and Tryptone soya agar plates then incubated at 37°C for 48 hours under 5-10% CO_2; and also on MacConkey agar and incubated for 24 h at 37°C under aerobic conditions. Colonies were identified based on morphology, culture character, and biochemical tests (Shonima *et al*., 2013).

Pathogenicity test

Suspected colonies were suspended in sterile PBS at concentration of 3×10^8 CFU/ml and inoculated in mice (0.2 ml intraperitoneal) and in duckling (age 9-10 days) (1ml intramuscular). The inoculated animals were kept under observation until developing of clinical signs or death for a period of 1week. Re-isolation of the organism was attempted from heart blood, lung, liver, spleen, and brain (Soman *et al*., 2014).

Identification of isolates by Polymerase Chain Reaction (PCR)

DNA extraction and purification carried on tissue homogenate of (liver, spleen and lung) or L.B broth by using Qiagen DNeasy Blood & Tissue extraction (Qiagen, Germany) and according to the manufacturer's protocol. Purified DNA recovered in 100-µl elution buffer and stored at -20°C for further testing. PCR primer pairs flanking about 608 bp of the *OmpA* gene of *R. anatipestifer* upstream primer (5'CTGCTCAGACTACT AGCAATC3') and downstream primer (5'GTTCAATG AAGCTGACGCTTG 3') (Biosearch Technologies South McDowell Boulevard Petaluma, CA, USA). PCR was carried out using a thermostable, proofreading DNA polymerase 4 x 1.25 ml DreamTaq Green PCR Master Mix (2X), which includes DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM MgCl2 cat No #K1081 and the PCR reaction was performed as following protocol: (25 µl 2× Dream Taq Green PCR Master Mix (Thermo Fisher Scientific Inc., MA, USA), 100 pmol represented in 1 µl for each forward and reverse primer, 5 µl template DNA, and nuclease-free water up to 50 µl. The thermal cycler was adjusted to initial 94°C for 5 min. cDNAs were then amplified with 40 cycles of 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 10 min (Heba *et al.,* 2015). The amplified products were analyzed by electrophoresis using 1% agarose gel and visualized by ultraviolet transilluminator after staining the gel with ethidium bromide stain (Fisher) as shown in (Fig.ure 2). The product size was measured using Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific) cat No #SM0323.

The universal primers of P. Multocida KMT1T7 –F(5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3') and KMT 1SP6-R (5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3') were used to exclude the presence of *P. Multocida* by detection of about 460 bp band. PCR reaction was performed as following protocol: (25 µl 2× Dream Taq Green PCR Master Mix (Fermentas), 100 pmol of each forward and reverse primers], 2 µl template DNA, and nuclease-free water up to 50 µl. The amplification reactions were performed using thermal cycler Perkin Elmer Gene Amp PCR system 9700. Cycling conditions

Table 1: The history of examined farms

Governorates	Examined farms	Breed	Age (day)	Morbidity %	Mortality %	Season
Qalubia	1	Pekin	19	60	8	Spring 2017
	2	Muscovy	15	40	13	Spring 2018
	3	Pekin	20	39	11	Autumn 2018
Beni-Sueif	4	Pekin	10	35	20	Spring 2017
	5	Muscovy	15	53	9	Autumn 2017
	6	Muscovy	7	48	12	Spring 2018
	7	Mallard	19	45	19	Autumn 2018
Fayoum	8	Mallard	16	38	20	Spring 2018
	9	Muscovy	7	47	7	Autumn 2018
Giza	10	Mallard	11	58	17	Autumn 2018

have been selected according to (Townsend *et al*., 1996) and was adjusted to initial denaturation at 95°C for 5 min, then 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by final extension at 72°C for 7 min. The positive control of the previously identified of *P. Multocida* PM/VSVRI/1962 vaccine strain was kindly supplied by the Aerobic Bacteria Vaccine Department, Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. Nuclease free water was used as PCR negative control as shown in (Fig.. 3).

Multiple sequence alignment

Sequencing was carried out by GATC Company, Germany by using ABI 3730xl DNA sequencer. Sequence analysis was carried out by multiple sequence alignment of the sequenced products carried out on both nucleotide and deduced amino acid sequences using data available on GenBank of the following sequences strain Th4 (MF459001.1), strain YXb1(MF458999.1), CH3 (CP006649.1), RA-CH-1 (CP003787.1), RA-GD (CP002562.1), strain SX (MF459000.1) , Yb2 strain (CP007204.1) , strain 17 (CP007503.1) , RA-CH-2 (CP004020.1) , strain RAf153 (JQ083167.1) ,strain 153 (CP007504.1) ,strain NCTC 11014 (LT906475.1), ATCC 11845 = DSM 15868 (CP003388.1), DSM 15868 (CP002346.1), HXb2 (CP011859.1) , strain RCAD0133 (CP029760.1) and GERD/VSVRI/Giza 2018 (MK587441) Multiple alignments, phylogenetic analysis and pairwise alignment of the deduced amino acid sequence against the reference OmpA/MotB domain family protein CDS Accession No # wp_015345393 carried out using Geneious 4.8. Program as shown in (Fig.ure 4).

RESULTS

Post-mortem findings

Postmortem examination revealed congestion of paranchymatous organs, pneumonia with presence of polyserositis in shape of fibrinous pericarditis; perihepatitis and air saculitis (Fig. 1A).

Bacterial identification

The morphological characters on blood agar plates yielded confluent grey moist convex, entire, transparent, non-hemolytic dew drop colonies after 48 h (Fig. 1B). The same morphological characters (confluent grey moist convex, entire, transparent, dew drop colonies) were shown on chocolate agar (Fig. 1C) and tryptone soya agar (Fig. 1D). There was no growth appeared on MacConkey agar (Fig.1E).

Bacterial smears from suspected colonies stained with Gram's staining (Fig. 1F) revealed small, Gram-negative cocci organisms arranged either singly or pairs, or in short chains when isolated from suspected organs but after subculturing on Tryptone soya broth gave cocco-bacillary, short rods to filamentous forms (Fig. 1G).

Smears from heart blood and liver impression stained with Leishmann stain revealed bipolar organisms. Biochemical tests shown that the organisms were catalase, oxidase, urease and gelatin liquefaction positive and showed that the isolates were negative for Indole, methyl red, citrate utilization and nitrate reduction tests. With regards to the fermentation of sugars, the isolates failed to ferment dextrose, galactose, lactose, fructose, mannose, maltose, mannitol, sorbitol, inositol, trehalose and sucrose.

Form 50 samples collected, there are 15 bacterial isolates were isolated from duck had lesions of fibrinous pericarditis; hepatitis; airsacculitis.

Pathogenicity test

Pathogenicity test revealed that mice is not susceptible to *R. anatipestifer* where all mice inoculated with suspected colonies later on identified as *R. anatipestifer* were healthy and did not die.

Pathogenicity test revealed that from 10 suspected bacterial isolates colonies there were 4 isolates able to make the disease picture in experimental infection in duckling died within 12-48 h while the control duckling inoculated with sterile PBS remained healthy. The gross lesions observed were enlargement and congestion of the liver and enlarged spleen (Fig. 1H). Heart blood and liver impression smears revealed bipolar organisms (Fig. 1I and 1J) when stained with Leishman's stain. *R. anatipestifer* could be re-isolated from heart blood, lung, liver, and spleen of the dead duckling's on 10% sheep blood agar.

Polymerase Chain Reaction

Four isolates out of 10 which were confirmed as *R. anatipestifer* by using specific primer flanking about 608 bp PCR product of outer membrane protein OmpA and made the disease picture in experimental infection in duckling which died within 12-48 h again after inoculation gave PCR product at the expected precise size while 6 isolates of 10 were PCR negative , The positive control previously identified and characterized was kindly supplied by Dr. Heba Naeim Deif, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. The positive PCR products (Fig. 2 lane 4) sent for sequence analysis and were analyzed by BLAST with isolates and strains exist on GenBank to assess the homology and identity percent.

The result of PCR carried out with universal primer of the of *P. Multocida* flanking 460 bp were negative and showed no amplification for the four selected samples (Fig. 3) to assure that causative agent of the epidemic picture reported in the nearby governorates was not due to *Pasteurella Multocida* as both diseases had very similar clinical symptoms.

Multiple sequence alignment of both nucleotides and corresponding amino acids based on the blast result of the local isolates with the available sequences on genbank of the corresponding sequenced fragment which revealed that there is single nucleotide substitution at the base pair number 500 from the start codon of the OmpA of *R. anatipestifer* which lead to single Amino Acid Substitution from ACA (Threonine(Thr/T)) which is considered as a polar Amino Acid to ATA (Isoleucine (ILe/I) which is a non polar Amino Acid which is very characteristic for the local isolate which is closely related to serotype1 that made the epidemic form of the disease in the nearby governorates from which the samples were collected (Fig. 3 and 4).

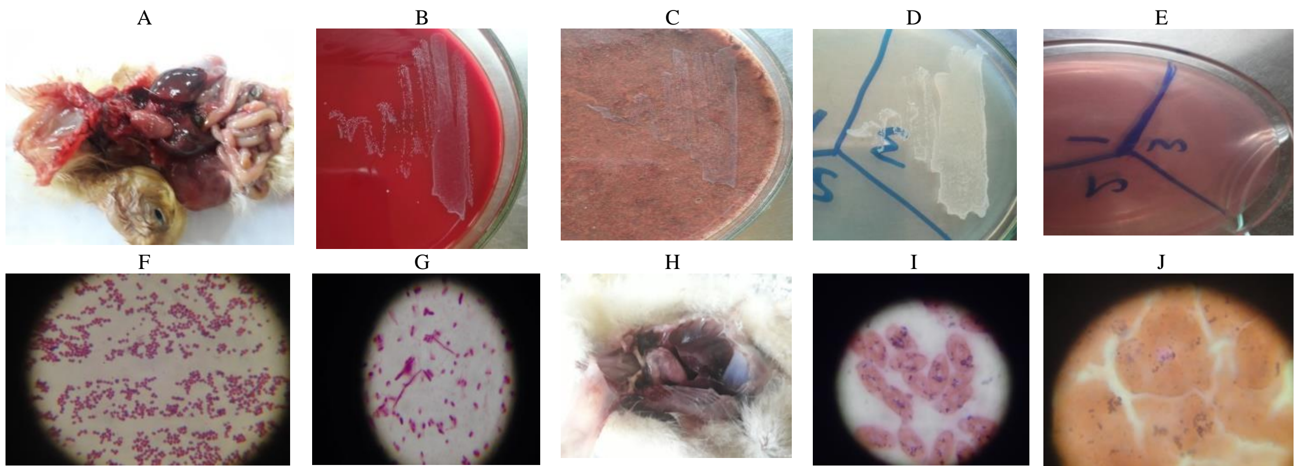

Fig. 1: Morphological picture (**A:** Postmortem examination showed pericarditis; airsacculitis; pneumonia, and hepatitis. **B:** grey, convex and non-hemolytic dew drop colonies on blood agar. **C:** Colony on chocolate agar. **D:** Colony on Tryptone soya agar. E: No growth on MacConkey agar. **F:** Gram's staining of the colonies revealed small, Gram-negative cocci organisms. **G:** Morphology varying from cocco-bacillary, short rods to filamentous forms after subculture. **H:** Experimentally infected duck showed congestion of liver and heart. **I:** Heart blood stained with Leishman's stain showed bipolar organisms. **J:** Liver impression smears stained with Leishman's stain showed bipolar organisms.

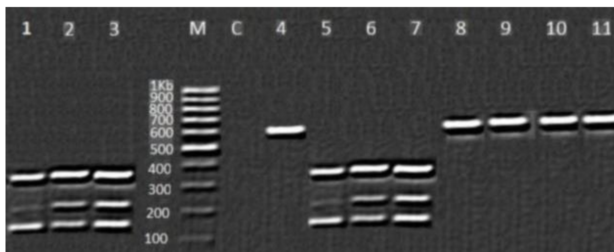

Fig. 2: PCR products carried on the control *Pasteurella multocida* as negative control (Lane C), positive control of reference *R. anatipestifer* strain (Lane 4), PCR products of 608 bp (Lane8,9,10,11) at the expected size of the field isolates including GERD/VSVRI/Giza 2018Accession No # MK587441 while Lane(1,2,3,5,6,7) negative PCR field isolates with nonspecific bands. Lane (M) Is100 bp DNA ladder.

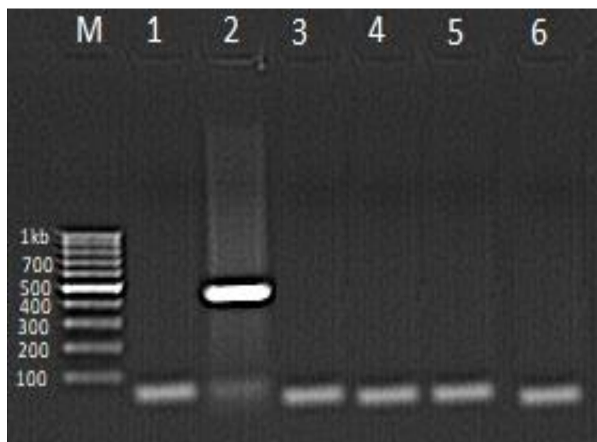

Fig. 3: PCR using the universal primer for detection of *P. Multocida* where the positive control is (lane 2) showing specific band at 460 bp while lanes from (3-6) showing negative result using the DNA of local isolates of *R. anatipestifer*, (lane1) is negative PCR control.

Pair wise alignments of 186 deduced amino acid residues of the outer membrane protein OmpA of the local isolate GERD/VSVRI/Giza 2018 Accession No # MK587441 against the reference OmpA family protein [Riemerella anatipestifer] Accession No # wp_015345393 showing change at 5 positions first position at residue number 26 which is corresponding to residue 46 from the start methionine of the OmpA from (Serine (S) to glycine (G)) and change at residue number 27 which is corresponding to residue 46 from the start methionine from (Alanine (A) to Valine (V)), and change at 76 which is corresponding to residue 96 from the start methionine from (Isoleucine (I) to Valine (V)) and another change at residue 84 which is corresponding to residue 104 from the start methionine from (Glycine(G) to Aspartate(D)) and the last change which is very characteristic for the local isolate GERD/VSVRI/ Giza 2018 than other isolates and strains at residue 167 which is corresponding to residue 187 from the start methionine from (Isoleucine (I) to Threonine (T)) as shown in (Fig. 5).

Phylogenetic analysis of 186 amino acid residue of the field isolate GERD/VSVRI/Giza 2018 Accession No # MK587441 revealed that local isolate is closely related and very similar to the *R. anatipestifer* CH3 strain Accession No #CP006649 which is classified as serotype 1, So the local isolate of interest in this study may belongs to serotype 1 which is the most prevalent serotype that caused the severity and the epidemic picture of the disease in the nearby governorates as shown in (Fig. 6). It has been recorded in several outbreaks around the world especially in Thailand and china that serotype1 is the most prevalent strain in several outbreaks and caused sever economic losses (Pathanasophon *et al*., 1995), while here in Egypt several fully characterized strains like strain HEB/NA-4 Accession No # KR809895,strain HEB/NA-3 Accession No # KR809894 and strain HEB/NA-1 Accession No # KR809892.1 (related to serotype 4) have been isolated from Qalubia governorate which is same the governorates where the strain GERD/VSVRI/Giza 2018 used in the current study has been isolated and strain HEB/NA-2 Accession No # KR809893.1which is related to serotype 3. In the current study OmpA of the isolate of interest is considered as keynote for the virulence of this

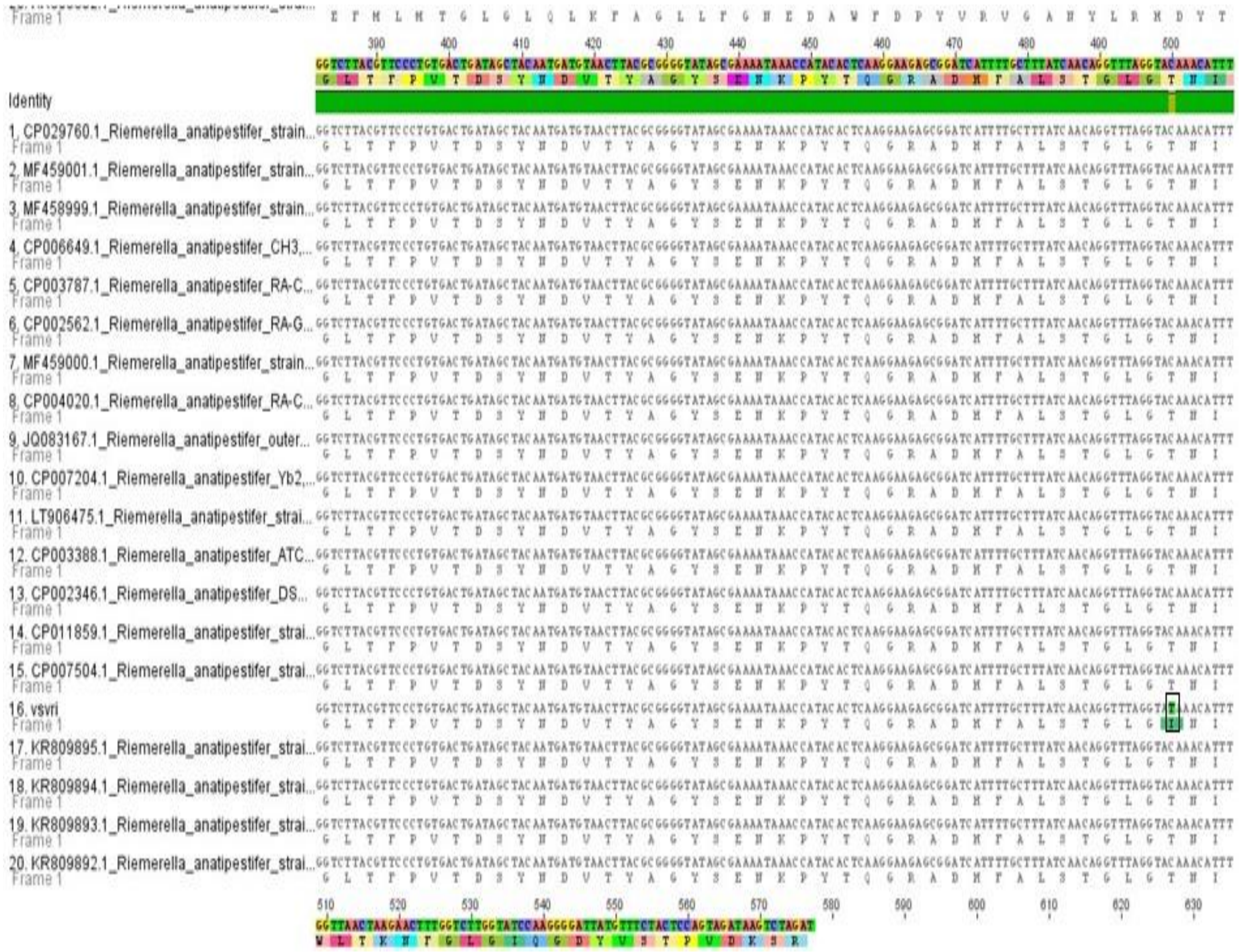

Fig. 4: Multiple sequence alignment of both nucleotides and corresponding amino acids of outer membrane protein OmpA of the local isolate GERD/VSVRI/Giza 2018Accession No # MK587441 with most of isolates and strains showing single nucleotide substitution at the base pair number 500 and the corresponding to amino acid 167 which is corresponding to the residue 187 from the Methionine (ATG) residue of the OmpA of the local isolate GERD/VSVRI/Giza 2018.

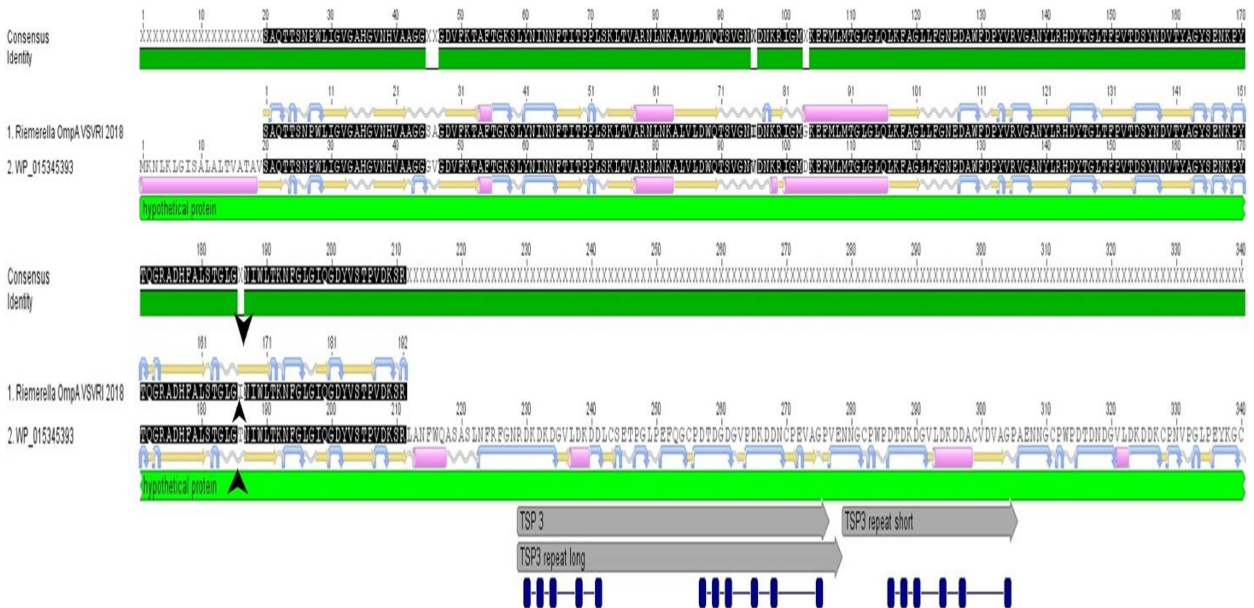

Fig. 5: OmpA/MotB domain family protein CDS showing schematic representation of alignment 0f 211 amino acid of outer membrane protein OmpA of the local isolate GERD/VSVRI/Giza 2018 Accession No # MK587441 against the reference OmpA/MotB domain family ,showing change at the residue number 167 which is corresponding to the residue 187 from the first methionine from (Threonine(Thr/T) which is considered as anon polar Amino Acid to (Isoleucine (ILe/I) of the reference OmpA family protein [*Riemerella anatipestifer*] Accession No # wp_015345393**;** https://www.ncbi.nlm.nih.gov/search/all/? term=wp_015345393.

Fig. 6: Phylogenetic analysis of the deduced amino acid sequence of outer membrane protein OmpA of the local isolate GERD/VSVRI/Giza 2018 Accession No # MK587441 using MEGA5 software this rooted tree was generated by neighbor-joining method with 1000 bootstrap of partial coding sequence of the outer membrane protein OmpA sequences. The bar represents a sequence divergence of 0.01 residue per site, the neighbor-joining method done by Saitou and Nei (1987).

serotype (serotype1) among the 21 serotype of *R. anatipestifer*, OmpA of *R. anatipestifer* is major immunogenic protein (Subramaniam *et al*., 2000) and plays an important role in virulence of the organism where mutated OmpA strains become attenuated and shows some sort of loss of adhesions to Vero cells which indicates that OmpA is an important virulence factor of *R. anatipestifer* (Hu *et al*., 2011).

DISCUSSION

Flavobacteriaceae, including the genus Riemerella, are Gram-negative bacteria, many of which possess relatively low fermentative activity. Due to this fact, identification and differentiation by standard biochemical methods is often difficult, leading to numerous misidentifications and incorrect classifications of strains. The history of the genus Riemerella is rich in examples of such misidentified isolates. The reference strains of the former *R. anatipestifer* serotypes 4 and 20 were later excluded from this species and subsequently replaced by new *R. anatipestifer* serotypes (Ryll and Hinz, 2000). Similarly, strain Coenonia anatina 1502T initially had been proposed to belong to *R. anatipestifer* (Vandamme *et al*., 1999). In addition, many isolates tentatively described as "Riemerella-like" bacteria based on biochemical characteristics, were by more detailed taxonomic analysis identified as only distantly related species, such as Pelistega europea, a member of the Proteobacteriaceae family (Vandamme *et al*., 1999).

Clinical signs included dullness, huddling, poor feed intake, refusal to swim, purulent oculo-nasal discharge, greenish diarrhea, incoordination and death. The gross lesions noticed like congestion of meninges, fibrinous pericarditis, perihepatitis, splenomegaly and airsacculitis (Kardos *et al*., 2007).

Surya *et al*. (2016) found that all the isolates were non-hemolytic on blood agar, except one (RA2), which produced a clear zone of haemolysis after 48 h of incubation, indicating it may be a different strain or serotype, since more than 20 serotypes of *R. anatipestifer* have been reported worldwide.

R. anatipestifer can be differentiated from *P. multocida* based on the inability of the former to produce indole and ornithine decarboxylase and its ability to liquefy gelatin (OIE, terrestrial manual, 2008).

Strains belonging to *R. anatipestifer* serotype 1 are responsible for most of the major disease outbreaks caused by this organism in ducks in Thailand (Pathanasophon *et al*., 1995)

The epidemic picture that has been recorded during spring 2017 and 2018 in very close governorates (Qalubia, Giza, Fayoum and Beni-Sueif) in a very short period of about one year and due to very similar epidemic picture in both clinical and postmortem finding with *P. multocida*. So, a rapid and sensitive method should be established to differentiate between the epidemic form of both diseases especially in the lack of specific hyperimmune sera and due to presence of about 21 serotype of *R. anatipestifer* which are not showing any cross protection between these serotypes (Subramaniam *et al*., 1997, Pathanasophon *et al*., 2002, Tsai *et al*., 2005) which is the major cause yet to develop effective vaccine to be protective against *R. anatipestifer*. The current study used PCR as a rapid, sensitive, specific, and simple method depending on detection of 608 bp as partial coding sequence of OmpA of *R. anatipestifer* due to its immunogenic importance and used as candidate for serotyping of *R. anatipestifer* depending on sequence analysis. The 4 selected isolates showed the same clinical picture after experimental infection and they were used for sequence analysis and multiple alignment. The 4 isolates showed 100% identity and homology which assures that the epidemic form of the disease may be caused by the same strain which been designed and submitted to Genbank as GERD/ VSVRI/Giza 2018 Accession No # MK587441. The sequence analysis of the isolate of interest revealed single nucleotide substitution at the base pair number 500 from the start codon(ATG) of the OmpA of *R. anatipestifer* which lead to single Amino Acid Substitution from ACA

(Threonine (Thr/T)) which is considered as a polar Amino Acid to ATA (Isoleucine (ILe/I) which is a non polar Amino Acid which is very characteristic for the local isolate and the phylogenetic analysis of 186 amino acid residue of the partial coding sequence so that local isolate is closely related and very similar to the *R. anatipestifer* CH3 strain Accession No #CP006649 which is classified as serotype 1. So, the local isolate of interest in this study may belong to serotype 1 which is the most prevalent serotype and caused the epidemic form of the disease in this very short period of time (spring 2017- spring 2018) in these very close governorates as shown in (Fig.ure 6). At the same time, it has been found from other sequences exist on Genbank and carried out on OmpA of *R. anatipestifer* in Egypt that the serotype 4 strain HEB/NA-1 Accession No # KR809892.1 exist in Qalubia governorate and the serotype 3 HEB/NA-2 Accession No # KR809893.1 exist in Giza governorate , which assures that may be more than one serotype may be found in the same district, Pair wise alignments of 186 deduced amino acid residues of the isolate of interest against the reference OmpA family protein [*Riemerella anatipestifer*] Accession No # wp_015345393 https://www.ncbi.nlm.nih.gov/search/all/?term=wp_015345393 revealed change in 5 residues along the sequenced part of the local isolate GERD/VSVRI/Giza 2018 shown in (Fig. 5) which may contribute to the virulence of the isolate of interest and made this epidemic picture.

Conclusions

Using PCR and sequence analysis of *Omp A* gene is considered a highly sensitive, rapid and an alternative method for serotyping of *R. anatipestifer* especially in case of unavailability of standard hyper immune serum. It is highly recommended to use the locally isolated *R. anatipestifer* (GERD/VSVRI/ Giza 2018) that belongs to serotype 1 – the most prevalent serotype in this study – for preparation of effective vaccine for controlling duck septicemia in Egypt. Also, further studies for continuous monitoring and surveying is recommended through Egyptian governorates to characterize the most prevalent strains to be used in polyvalent vaccine formulation due to absence of cross protection between the 21 serotypes of *R. anatipestifer*.

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