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Genome Sequences of Gcp Gene of *Mannheimia haemolytica* Serotypes A1 and A2 Associated with Respiratory Manifestation of Ruminant in Egypt

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Abstract: *Mannheimia haemolytica* is the most common bacterial isolates that cause pulmonary diseases in ruminants. This study examined the molecular characteristics and genome sequencing of virulence-related O-sialoglycoprotease (gcp) gene of *Mannheimia haemolytica* isolated from cattle, buffaloes, sheep and goats suffered from respiratory manifestations from which148 samples were collected as 56 nasal swabs and 92 lung tissues for bacteriological examination of *Mannheimia haemolytica* then identified morphologically, microscopically and biochemically. Only 20 biochemically typical *Mannheimia haemolytica* colonies were obtained; then tested by Polymerase Chain Reaction (PCR) for the presence of gcp gene which gave positive bands at 227bp in only 2 yielded colonies, one of them yielded from1 cattle nasal swab and the other recovered from1 sheep lung tissue, followed by genome sequencing of gcp gene of both PCR-positive yielded isolates, the genome sequencing of gcp gene explored they were *Mannheimia haemolytica* serotype A1 and serotype A2, respectively, while the rest 18 biochemically typical *Mannheimia haemolytica* yielded isolates gave PCR-negative bands, these may suggested that the absence of gcp gene in PCR-negative isolates or they have divergent sequences that were not amplifiable by the PCR assays and may provide an advantage PCR-positive 2 isolates during infection. Genome sequencing confirmed that *Mannheimia haemolytica* serotype A1 is the most prevalent in cattle while serotype A2 most common in sheep suffering from respiratory manifestation.

Key words: *Mannheimia haemolytica* · Genome Sequence · Gcp gene · Ruminant

INTRODUCTION

Mannheimia (Pasteurella) haemolytica is the etiologic agent of pneumonic pasteurellosis of cattle and sheep, which is infectious and may cause considerable economic losses to the cattle and sheep industries, *Mannheimia haemolytica* species are 12 identifiable serotypes in which there is apparent host specificity in disease manifestation; serotype A1 is the most common serotype isolated from cases of bovine pneumonia whereas A2 is the predominant isolate of ovine pneumonia and systemic disease [1, 2]. *Mannheimia haemolytica* is the principal bacterial pathogen associated with bovine respiratory disease (BRD). As an opportunistic pathogen, *M. haemolytica* is also frequently isolated from the respiratory tract of healthy cattle [3] and in Egypt; it can be obtained from the upper respiratory tract of both apparently healthy and diseased cattle, buffaloes, sheep and goats [4].

Potential virulence factors of *M. haemolytica* have been identified and characterized by gene cloning and DNA sequence analyses [5]. These factors include a ruminant-specific leukotoxin, an anti-phagocytic capsule, lipopolysaccharide, iron-restricted outer membrane proteins, a sialoglycoprotease, a neuraminidase and immunoglobulin proteases. Since *M. haemolytica* undergoes a niche change from commensal to pathogenic, the control of its virulence factor expression is also of significant interest. It has been shown that *M. haemolytica* exhibits a system 2 quorum-sensing mechanism to regulate gene expression under specific conditions [6].

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The genome sequence of *M. haemolytica* was recently published [7] under the direction of Dr Sarah Highlander of Baylor College of Medicine, Houston, Texas with funding provided by the USDA National Research Grant Initiative 00-35204-9229. The *M. haemolytica* genome consists of a DNA with an approximate G & C content of 41%. The genome length is about 2.57Mb. The annotated genome includes 2839 coding sequences, 1966 of which were assigned a function and 436 of which are unique to *M. haemolytica*. Through genome annotation many features of interest were identified, including bacteriophages (ÔMhaMu1 and ÔMhaMu2) and genes related to virulence, natural competence and transcriptional regulation. Comparison of competence loci and DNA uptake signal sequences (USS) in other species in the family *Pasteurellaceae* indicates that *M. haemolytica, Actinobacillus pleuropneumoniae* and *Haemophilus ducreyi* form a lineage distinct from other *Pasteurellaceae*. This observation was supported by a phylogenetic analysis using sequences of predicted housekeeping genes [7]. The whole genome shortgun project has been deposited at GenBank under project accession number AASA00000000 while the draft version has accession number AASA01000000.

MATERIALS AND METHODS

Samples: Nasal swabs and lung tissues (148 samples) have been collected from 54 cattle, 25 buffaloes, 42 sheep and 27 goats suffering from respiratory manifestation, freshly dead and freshly slaughtered ruminants from different localities in Alexandria and El-Bohira governorates during (2013-2014). Samples transported onto transport medium in an ice box within 1-3 hours to lab. for bacteriological isolation, microscopical and biochemical identification of *Mannheimia haemolytica* [8, 9] and molecular identification of *Mannheimia haemolytica* virulence related gene O-sialoglycoprotease (gcp) at 227bp by using the followed primer: gcp F 5'-CGT TGG GCA ATA CGA ACT AC-3' and gcp R 5'-GGC TTT AAT CGT ATT CGC AGC-3' [10], then genome sequences of gcp gene of PCR- positive *Mannheimia haemolytica*.

Agarose Gel Electrophoresis:

Sample Loading and Visualization: The PCR products were loaded in agarose gel to determine the base pairs of the PCR product which could be visualized by the presence of marker (Fermentas) and using Gel documentation system (Biometra).

Table 1: Thermal profile used in sequencing reaction:

Cycle	Time	Temperature
1 cycle	1 min	96°C
25 Cycles	10 sec	96°C
	5 sec	50°C
	2 Min	60°C

Method for Purification of the Pcr Products:

QIaquick Gel Extraction Kit Protocol: It was done using QIA quick gel extraction kit. (Qiagen, GmbH, Hilden Germany):

Sequencing reaction:

Procedure: Using Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA) cat-number 4336817according to the instruction of the manufacture as follows:

Sequence Analysis of the Amplified Fragment: Several sequence manipulation and analysis options and links to external analysis programs facilitate a working environment which allows you to view and manipulate sequences with simple point-and-click operations [11] and MEGA 4.0.2 software [12]. Sequence submission was conducted following the instructions offered by the web tool BankIt of GenBank. (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

RESULTS AND DISCUSSION

Bacteriological examination of 148 samples collected from cattle, buffaloes, sheep and goats suffering from respiratory manifestation revealed the isolation of 20 (13.5%) biochemically typical *M. haemolytica*. The incidence rate of *M. haemolytica* in cattle was 7(13%), buffaloes 2 (8%), sheep 6 (14.3%) and goats 5(18.5%), these were nearly similar to results of Frank [15] in United States who found that 15.8% of sheep nasal exudates yielded *M. haemolytica* and lower than Dawari *et al.* [14], who isolated *M. haemolytica* from 4 (57.14%) sheep and 1 (25%) from goats, Wray and Thompson [16] who mentioned that the prevalence of *M. haemolytica* found in calf nasal exudates was (87.70%), also Kaoud *et al.* [4] in Egypt who investigated the occurrence of *M. haemolytica* in buffaloes, sheep and goats with percentages of 20%, 22.20% and 26.70%, respectively and higher than that of Ilhan and Keles [17] who published that out of 584 lung samples of slaughtered sheep having clinical symptoms of pneumonia, 66 (11.35%) *M. haemolytica* strains were isolated and Kaoud *et al.* [4] who recorded that the

incidence of *M. haemolytica* in the diseased Egyptian cattle was 8%. This may suggested the influence of bacterial factors, virulence, localities and season effects in epidemiology of *M. haemolytica* infection in ruminants.

Molecular identification of biochemically typical *M. haemolytica* revealed that, of them, only 2(10%) gave PCR-positive bands at 227bp of virulence-related gcp gene, this may be due to that the absence of gcp gene in PCR-negative isolates or they have divergent sequences that were not amplifiable by the PCR assays and may provide an advantage PCR-positive 2 isolates during infection. Extensive epidemiological studies of *M. haemolytica* strains using molecular typing techniques show some significant level of heterogeneity even within strains of the same serovars isolated from various hosts and also from different geographical areas [18- 20].

Data in Fig. (1-3) illustrated the result of partial coding of gene sequencing of virulence-related gcp gene in the two yielded PCR-positive *Mannheimia haemolytica* strains done in MACROGEN, KOREA stated that one of them is *Mannheimia haemolytic* serotype A1 which isolated from cattle nasal swap and the other is *Mannheimia haemolytic* serotype A2 which isolated from sheep lung tissue, this agreed with Frank, Gilmour and Gilmour, Richard *et al.* and Sanchis *et al.* [1, 2, 21, 23]; who published that Serotype A1 is more prevalent in cattle while serotype A2 is more prevalent in sheep and disagreed with Kodjo *et al.*[18] and Chaslus-Dancla *et al*. [24] and Fodor *et al*. [25] who stated that in some cases, strains belonging to the same serotype have been found in various animal hosts, i.e. serotype A1 in bovine, ovine and caprine hosts. So determining the relatedness of a group of bacterial isolates is essential for epidemiological investigations [26].

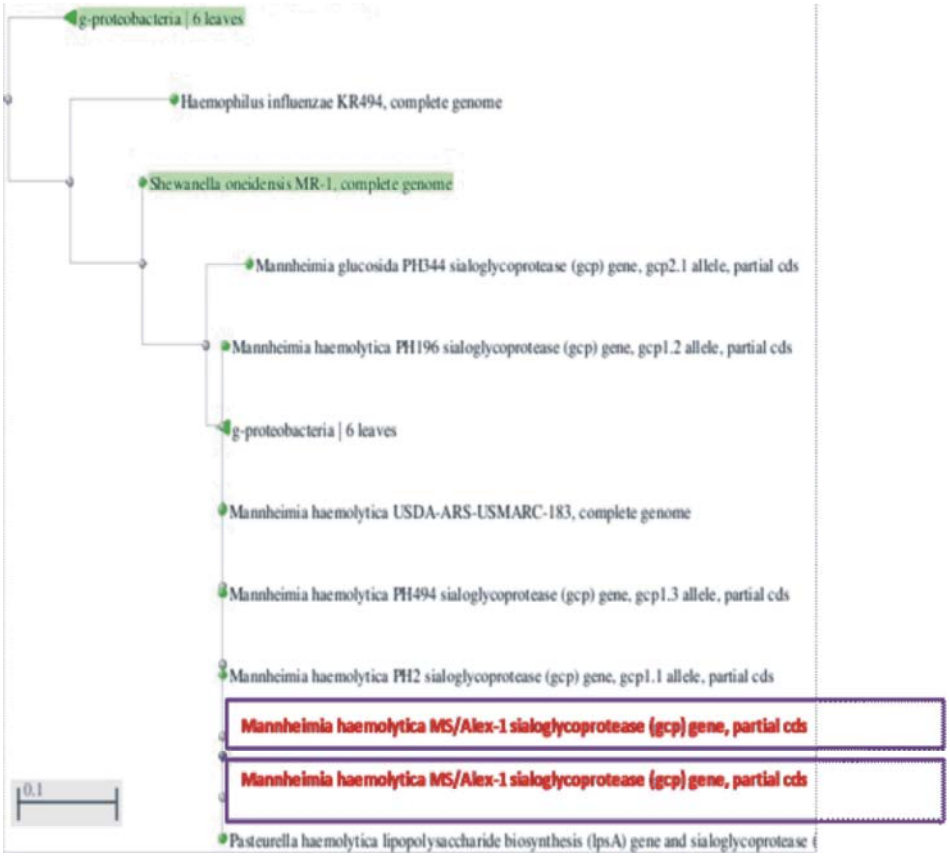


Fig 1.1: The Neighbor joining Phylogenetic rooted tree of partial O-sialoglycoprotease (gcp) gene based on nucleotide sequence showing the clustering of *Mannheimia haemolytica* MS/Alex-1 and *Mannheimia haemolytica* MS/Alex-2 strain with other representative *Mannheimia haemolytica* genetic groups. The numbers at the nodes represent bootstrap values. Scale bar represents the number of substitutions per site. The year of isolation and geographical origin of the bacteria sequences are included in the tree. The tree was generated by Mega4 software program.

MS/Alex: Mohamed Saed/Alexandria.

Fig 1.2: Neighbor joining Phylogenetic rooted tree of partial O-sialoglycoprotease (gcp) gene of Egyptian *Mannheimia haemolytica*.

MS/Alex: Mohamed Saed/Alexandria.

Fig 1.3: Neighbor joining Phylogenetic rooted tree of partial O-sialoglycoprotease (gcp) gene of Egyptian *Mannheimia haemolytica*.

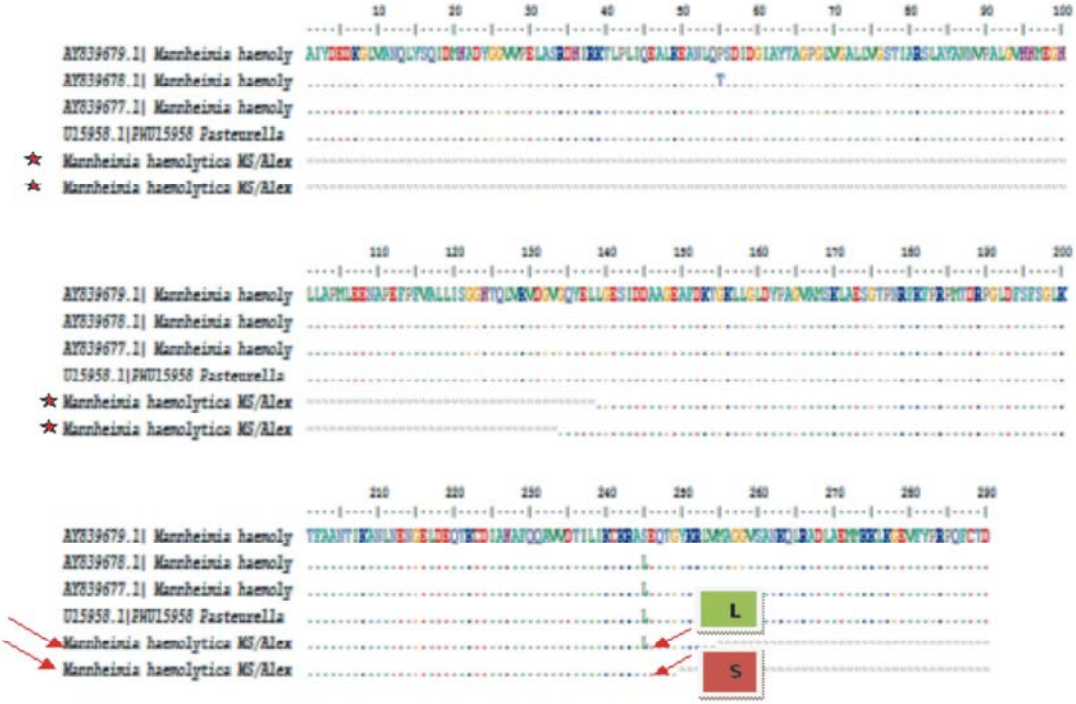
MS/Alex: Mohamed Saed/Alexandria.

Fig 2: Multiple nucleotide sequences alignment of partial O-sialoglycoprotease (gcp) gene of *Mannheimia haemolytica* MS/Alex-1 and *Mannheimia haemolytica* MS/Alex-2strains in comparison with other representative circulating strains. The Dot (.) represents identity whereas single alphabet represents difference in the nucleotide sequence.

Table 2: Identity Percentages of partial O-sialoglycoprotease (gcp) gene of recent Egyptian *Mannheimia haemolytica* strains:

		Identity percentage					
Strain	No.	1	2	3	4	5	6
Mannheimia haemolytica PH196	1	ID	100%	100%	100%	40%	40%
Mannheimia haemolytica PH494	2	100%	ID	100%	100%	40%	40%
Mannheimia haemolytica PH2	3	100%	100%	ID	100%	40%	40%
Pasteurella haemolytica lipopolysaccharide biosynthesis	4	100%	100%	100%	ID	40%	40%
Mannheimia haemolytica MS/Alex-1	5	40%	40%	40%	40%	ID	91%
Mannheimia haemolytica MS/Alex-2	6	40%	40%	40%	40%	91%	ID

ID: means identical. MS/Alex: Mohamed Saed/Alexandria



MS/Alex: Mohamed Saed/Alexandria.

Fig 3: Amino acid alignment of partial O-sialoglycoprotease (gcp) gene of *Mannheimia haemolytica* MS/Alex-1 and *Mannheimia haemolytica* MS/Alex-2 strains in comparison with other representative *Mannheimia haemolytica* circulating strains. The Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence.

Nucleotide Sequence Accession Numbers: The partial genome sequences of *M. haemolytica* O-sialoglycoprotease (gcp) gene of Serotype A1 and A2 were deposited in GenBank under accession numbers: BankIt1789308 Seq1 KP411385 for *Mannheimia haemolytica* MS/Alex-1 sialoglycoprotease (gcp) gene, partial cds and BankIt1789312 Seq1 KP411386 for *Mannheimia haemolytica* MS/Alex-2 sialoglycoprotease (gcp) gene, partial cds; respectively.

Multiple Nucleotide Sequence Alignment: The obtained sequence with the highly similar sequences in the blast result were downloaded and imported in BIOEDIT version 7.0.4.1 multiple sequence alignment were conducted using Clustal W application embedded in the BIOEDIT. Multiple nucleotide sequence alignment revealed unique nucleotide substitution was recognized, to further identify the genetic characteristics of Egyptian *Mannheimia haemolytica*, partial nucleotide sequence of partial sialoglycoprotease (gcp) gene of *Mannheimia haemolytica* MS/Alex-1 and *Mannheimia haemolytica* MS/Alex-2 was carried out and compared with other representative *Mannheimia haemolytica* circulating strains which are available on GenBank.

Amino Acid Alignment: Deduced amino acid sequences were aligned using BIOEDIT 7.0.4.1 Clustal W application. Multiple amino acids alignment revealed the presence of prominent amino acid substitutions. Results are shown below:

Phylogenetic Analysis: The goal is to assemble a phylogenetic tree representing a hypothesis about the evolutionary ancestry of a set of genes, species, or other taxa. Phylogenetic trees generated by computational phylogenetic can be either rooted or unrooted depending on the input data and the algorithm used. A rooted tree is a directed graph that explicitly identifies a most recent common ancestor (MRCA), usually an imputed sequence that is not represented in the input. Identification of a root usually requires the inclusion in the input data of at least one "outgroup" known to be only distantly related to the sequences of interest. Phylogenetic analysis of the newly obtained nucleotide sequences in this study using MEGA 4.0.2 software. The evolutionary history was inferred using the neighbor-joining method and the reliability of each tree branch was estimated by performing 1000 bootstrap replicates [12]. A phylogenetic tree was constructed for better understanding the genetic relatedness and evolution of recent Egyptian *Mannheimia haemolytica* isolates.

The Bacteria Isolated in this Study Were Designated As: *Mannheimia haemolytica* MS/Alex-1 and *Mannheimia haemolytica* MS/Alex-2.

CONCLUSION

Mannheimia haemolytica is most commonly associated with respiratory manifestation in ruminant, PCR assay for detection of virulence related gene O-sialoglycoprotease (gcp) gene could suggested its pathogenicity, rapid, sensitive, specific, facilitate identification and does not require laboratory animal, genome sequencing for gcp gene confirmed that *Mannheimia haemolytica* serotype A1 is most prevalent in cattle while serotype A2 most common in sheep suffering from respiratory manifestation. These could help in determining epidemiology, bacterial factor and genetic basis of virulence and pathogenesis, control in outbreaks and development of the bovine respiratory disease and could also provide potential candidates for serotype-specific vaccine development against *Mannheimia haemolytica.* Genome sequencing can look at millions of transcripts in a single run and determine relative expression levels of individual genes.

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