Molecular characterization of *Avibacterium paragallinarum* strains used in evaluation of coryza vaccine in Egypt.

Amal, M. El-sawah; Y.A. Soliman and S.M. Shafey

Central Laboratory for Evaluation of Veterinary biologics, Abbasia, Cairo, Egypt
yousefadel00@hotmail.com

Abstract: In the present study, several molecular techniques were used to analyze the two serovars (A and C) that used in the quality control assays of Avibacterium ***paragallinarum*** (***A. paragallinarum***) vaccines attained to our laboratory (Central Laboratory For Evaluation Of Veterinary Biologics CLEVB). Western blotting analysis clearly revealed a differences in bands intensity when reacted to antisera prepared against either serovar A or C especially at area of 40-55 KDa. On the other hand nucleotide sequence analysis could revealed three single nucleotide polymorphisms (SNPs) between serovar A and C at position of 17(T/C) , 46 (G/A) and 178 (T/C) and one area of deletion in serovar C at nucleotide position 94 – 102. Hence these findings represent a good molecular marker for conformity and differentiation between the two tested serovars.
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1. Introduction

The Gram-negative nonmotile polymorphic ***Avibacterium paragallinarum*** (formerly classified as ***Haemophilus paragallinarun***; causes infectious coryza disease which is an acute respiratory infection of breeders, laying hens, and broiler chickens (**Blackall, et al., 2005**). Infectious coryza occurs worldwide, and causes significant economic losses through increased culls and a marked drop in egg production (from 10% to more than 40%) (**Blackall, 1999**).

There are two different but related serotyping schemes for ***A. paragallinarum*** have been mainly used. The Page (***Page, 1962***) and the Kume (**Kume et al., 1983**) schemes are both performed using haemagglutination inhibition (HI) tests (**Blackall & Yamamoto, 1998**) schemes which depend mainly on the haemagglutinin antigen. The Page scheme was initially developed by using a plate or slide agglutination test to recognize the three serovars, A, B, and C (**Page, 1962**). However, the use of haemagglutination-inhibition (HI) technology has been shown to be a much better method for identifying the Page serovar of field isolates of ***A. paragallinarum*** (**Blackall et al., 1990**). It is widely accepted that the three Page serovars represent distinct "immunovars," since inactivated vaccines based on any one Page serovar provide no cross protection against the other two Page serovars. It is generally accepted that cross-protection occurs only within a Page serovar (**Blackall et al., 1997**). The Kume serotyping scheme was originally based on hemagglutination- inhibition tests that recognized seven serovars organized into three serogroups termed I, II, and III (**Kume et al., 1983**). Subsequent publications have reported the existence of two further serovars and the recognition that the three Kume serogroups correspond to the three Page serovars. Hence, the reorganized Kume scheme now recognizes three serogroups (termed A, B, and C) which correspond to the Page serovars, with four serovars being recognized within both Kume serogroups A and C (**Blackall et al., 1990**).

Within page serogroup, inactivated vaccine prepared from one page serovar protect only against homologous challenge while Bivalent vaccines contain page serovars A and C can provide protection against page serovar B **(Blackall, 1999).** These difficulties have resulted in at least one commercial vaccine that contains multiple page serovar B strains to provided better protection. On the other hand within Kume serogroup A , serovas A-1 ,A-2, A-3 are strongly cross protective , while there is good cross protection between serovars A-1 andA-4.with in Kume serogroups C , there was a good level of cross protection for serovar C-1,C-2,C-3.

It worth mentioned that several studies have mentioned the role of haemagglutinin antigen in the protective immunity. Treatment of chicken with monoclonal antibodies against HA antigen protect them against challenge with the live ***A. paragallinarum*** strain and so reduced the load of such microorganism in the sinuses of the chicken (**Takagi et al., 1991**)

In our Laboratory (CLEVB) the evaluation of the infectious coryza inactivated vaccine depends on the challenge test using both serovars (A and C) after vaccination of the experimental birds, so the

current study aimed to molecular characterization of ***A. paragallinarum*** serovar A and C that used for evaluation procedures of such vaccines.

2. Materials and Methods

Strains:

Avibacterium paragallinarum serotypes A and C seovar were obtained from Reference culture bacterial strain unit of CLEVB, Abassia Cairo. Each strain was grown separately on chocolate agar supplemented with 0.25 % NADH (Nicotinamide adenine dinucleotide) in 5 % CO2 atmosphere for 48 hrs. (**Blackall and Yamamoto 1998**). A single colony was then picked up and grown in brain heart infusion broth supplemented with 1% sterile chicken serum and 25 µg / ml of sterile NADH. After 48 h of incubation at 37°C as before, a drop of the media was placed on a slide and stained with grams stain to check for the purity. Cells were then harvested by centrifugation at 4.800rpm/15min/4°C washed twice with cold PBS pH 7.2 and stored at -20°C till used.

Biochemical reaction :-

Biochemical reaction was done using API 20E strips (Biomriax cat 1000/97580) and the strains were tested for sugar fermentation, catalase, urease, and Indol production as recorded by **Mackie and macCarteny *(1996)*.**

Dual extraction of the genomic DNA and protein:

Both genomic DNA and bacterial proteins were extracted from a 0.5 mL sample of each strain (**Sambrook et al., 1989*),*** using Trizol reagent (Life technology cat # 15596) according to the instruction of the manufacture .

First, 0.5 ml of Trizol reagent was added to 0.5 ml of each strain, and incubated at 25°C/30min, and then 200µl of chloroform was added and incubated for 3 min before centrifugation at 14000rpm /15min at 4°C. The upper aqueous phase was completely removed; the DNA in the interphas was precipitated by adding 0.5ml of absolute ethanol and centrifuged as before.

Extraction and purification of genomic DNA:

The precipitated DNA from the interphas washed twice with 0.1M sod. citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml HEPES (0.1 M). Two µl of RNAase were then added and incubated at 37°C for 1 hour. Purification of the genomic DNA was done using Wizard DNA clean up system (Promega). The DNA was then analysed by agarose gel electrophoresis on 1% agarose and visualised using the UV transilluminatore.

Extraction and purification of bacterial proteins:

The protein rich supernatant was transferred to another 1.5 microfuge tube and the protein was precipitated with double volume isopropyl alcohol and sedimented by centrifugation as before. Protein was then washed twice with 0.3 M guanidine hydrochloride in 95% ethanol, centrifuged as before. The protein pellet was then redissolved in 100µl of PBS and stored at -20°C till used.

Determination of protein concentration:

The protein concentration was estimated using modified lowery method (**Ohnishi and Barr, 1978).**

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS -PAGE):

Extracted proteins of each strain were resolved on discontinuous buffer system composed of 10% (w/v) acrylamide separating gel and 4% stacking gel (**Laemmli, 1970*).*** Electrophoresis was carried out at a constant voltage (100 V) until the bromophenol blue dye moved to the bottom of the gel. The gels were stained with coomassie brilliant blue for 2 hours and destained overnight. Molecular weight of each protein band was calculated with reference to a standard curve derived from the migration pattern of standard prestained molecular weight markers (Page Ruler, Fermentas cat#SM0671).

Western blotting analysis:

The electrophoretic transfer of polyacrylamide gel resolved proteins to the nitrocellulose membrane was carried out by electroblotting as described (**Towbin et al., 1979*)*** using BioRad Electro Transfere unit. The unoccupied sites on the nitrocellulose membrane were blocked with blocking buffer (Tris buffered saline TBS, pH 7.2 containing 0.1% Tween-20, 1% (w/v) western blot grade gelatin and 0.05% Triton X100). The nitrocellulose membrane was then incubated with chicken Anti ***A. paragallinarum*** serovar A or C antibodies (1:500 in blocking buffer) at 37°C for 1 hour followed by washing three times with TBS-Tween 20. The membrane was then incubated at 37°C for 1 hour in anti-chicken peroxidase labeled dilution of 1:5000 in TBS- tween 20. The membrane was then washed as above and incubated in freshly prepared substrate solution (10 mg aminoethyle carbazone in 2.5 ml Isoamyleformamide and 47.5 mL of acetate buffer pH 5to which 50 µl of 30 % H_2O_2 was added) for 3-4 min for color development and visible bands were developed, then the reaction was stopped by washing the membrane with running distilled water. Page Ruler prestained protein ladder (Fermentas cat#SM0671) was used in this experiment.

PCR amplification:

A primer was designed to amplify the heamagglutinin gene of ***A. paragallinarum*** using DNASTAR® V9 software. The PCR was performed in 50-µl reaction mixtures containing 25µL of green Dream Taq master mix (fermentase Cat # K 1081) and 50 pmol of the forward primer (5'-

AAGCTTTTATTTTAGATTTATTG- 3') and the reverse primer (5'-CTGCTTGCACTAAGCCGTTG-3'). Thermal cycling was performed using T professional , thermal cycler (Biometra , Germany), the parameters for amplification were denaturation at 95 ^{o}C for 3 min and 40 cycles at 95^{o}C for 1 min, 54 ^{o}C for 45 sec, and 74^{o}C for 1 min. A final extension at 72 ^{o}C for 10 min was also included. The amplicon was electrophoresed on 1% agarose. 100pb DNA ladder (100 pb ladder , SibEnzyme Cat# M25) was used to calculate the exact amplicon size

Sequencing:

The complete nucleotide sequences of the haemagglutinin gene of the two serovars were performed in (Macrogen USA). For preparation of the gene for sequencing, the PCR product was separated on 1% low melting agarose and electrophoresed on low voltage (20 volt) at 4°C. The bands were sliced off and purified with the Biospin PCR purification kit (Biobasic cat # BSC03S1) as described by the manufacture. Briefly, the gel slices were melted at 60°C for 5 min, mixed with 500 ml of gel extraction buffer and placed on the biospin column provided with the kit, centrifuged at 4000 rpm/2min/4°C and washed twice with the washing solution. Finally the amplicon was eluted in 50 µL of the elution buffer and stored at -20 °C till sequenced. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Analysis:

The SDS PAGE and western blotting were analyzed with BioDocAnalyze version 2.66.3.1. The sequence analysis done with either Lasergene DNASTARE version 9 or CLC main workbench version 6.5.

3. Results

Strains identification :

After the incubation period, small grayish colonies were observed. Gram-negative, filamentous, pleomorphic bacteria were identified on microscopic observation and was typical for ***A. paragallinarum***. Biochemical check (photo 1) gave positive results identical for ***A. paragallinarum***

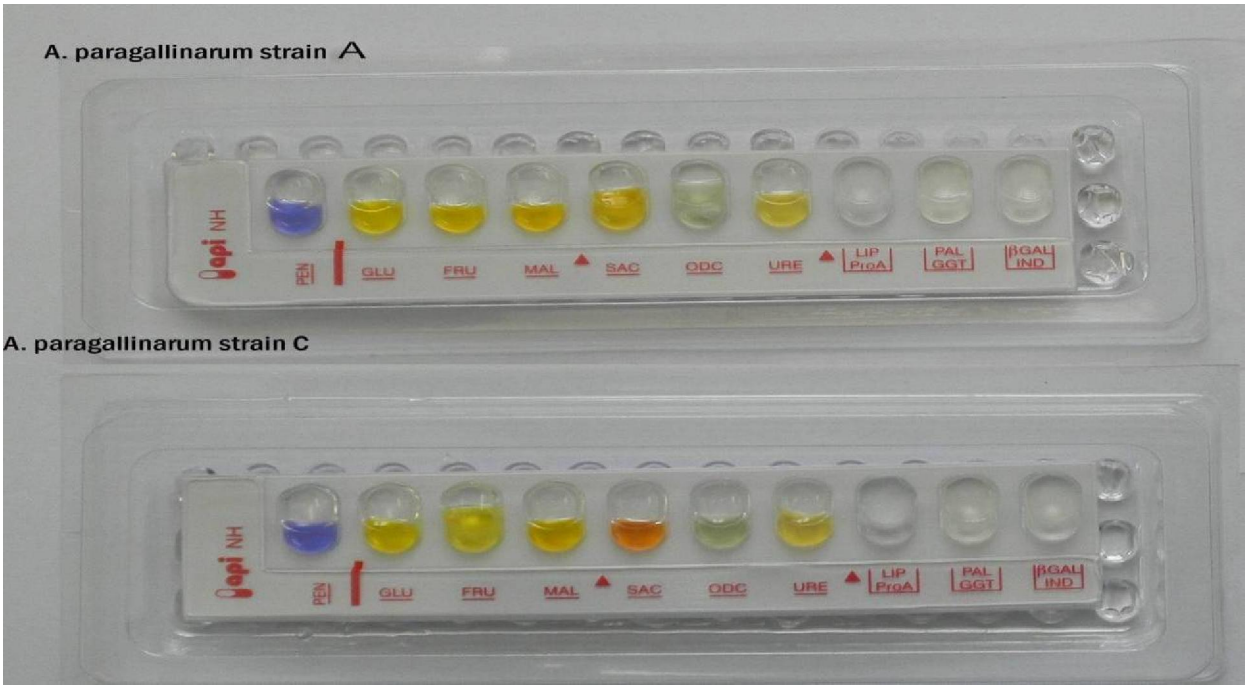


Photo (1) the biochemical identification of the *A. paragallinarum* strain A and C. note the complete match of the biochemical reaction to the Avibacterium spp.

SDS page analysis:

SDS analysis of the protein purified from both strains gave nearly identical pattern with more clear visible bands grouped between 70-100 KDa, 40-55KDa and 25-30 KDa. These identical bands have a limited degree of intensity indicating different bands concentrations although the total protein used in the electrophoresis was the same.

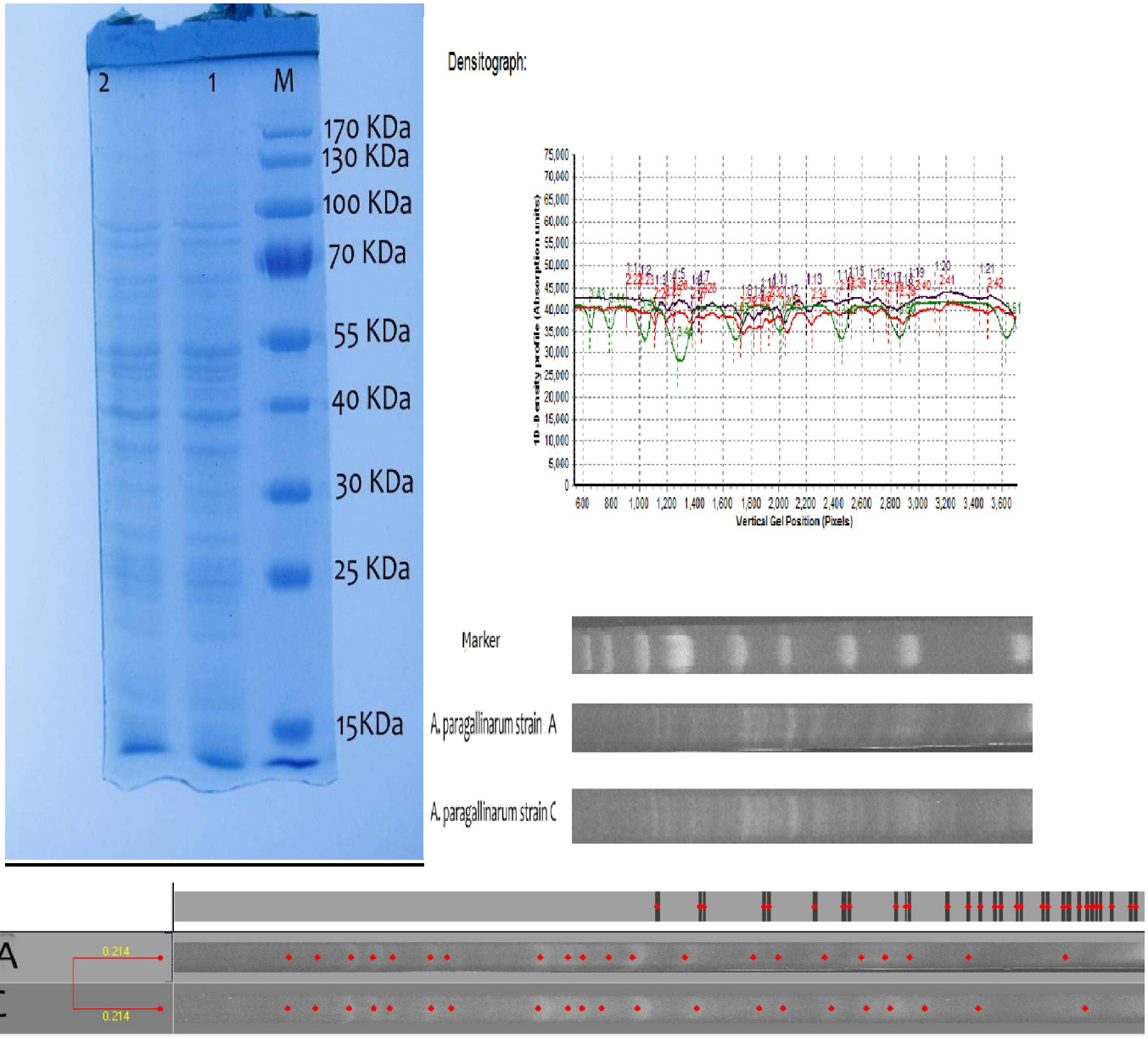


Fig (1) SDS page analysis (upper left) of *A. paragallinarum* strain A (lane 1) and strain C (lane 2), the densitgraph analysis generated by BioDoc analysis software (upper right) and The similarity index using cluster NJ method (lower)

Western blotting analysis of A. paragallinarum:

The western blotting analysis of the electrotransfered gels of both strains gave also the same pattern. But it was noticed that the bands between 40-55KDa reacted differently to the A or C antisera. When using the **A. paragallinarum** strain B antisera, the 45KDa bands reacted more strongly than using the **A. paragallinarum** strain B antisera, on contrary to the band migrating at 48KDa which reacted more strongly with the antisera against **A. paragallinarum strain C.**

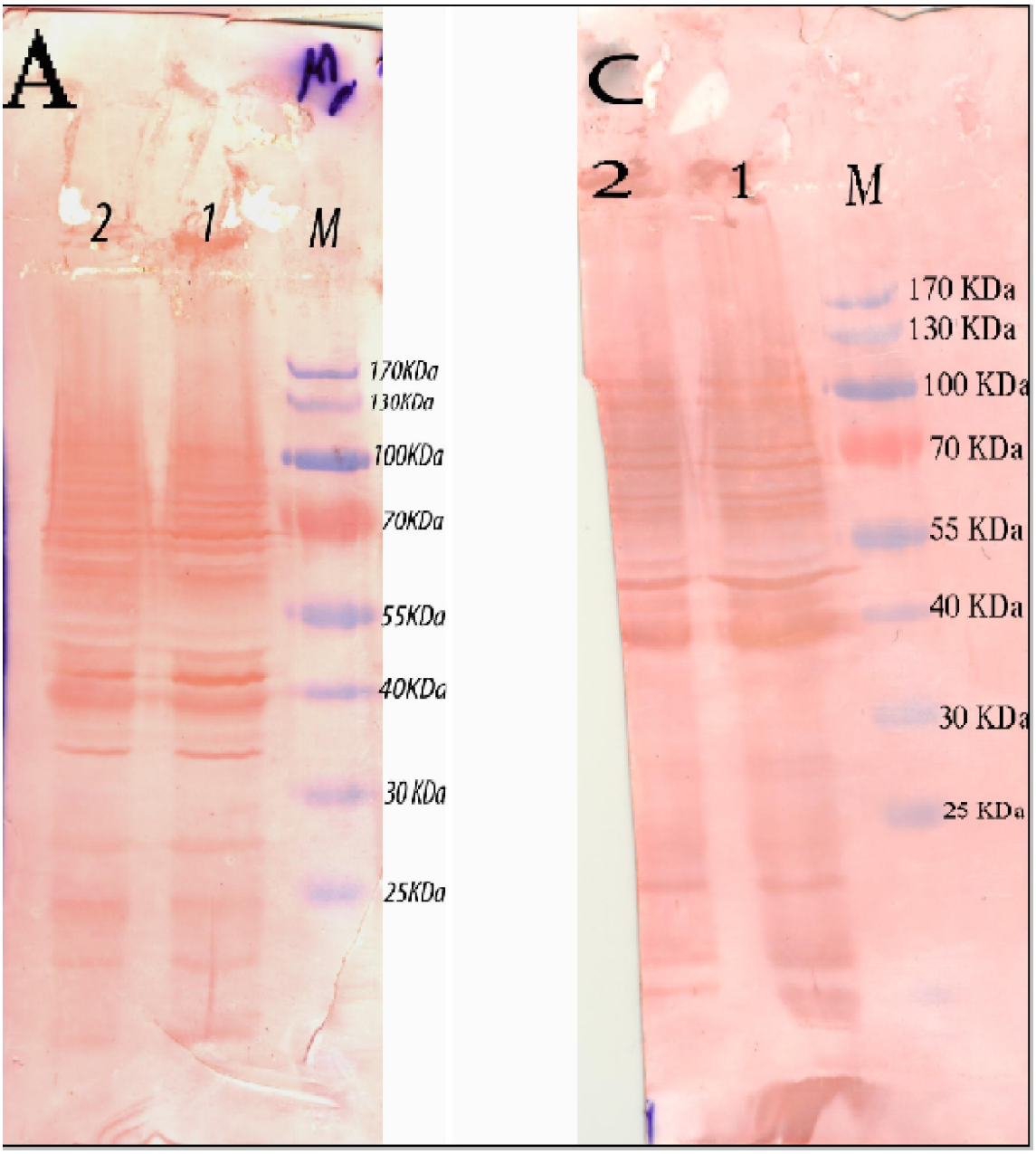


Fig (2) western blotting analysis of *A. paragallinarum* strain A (lane 1) and strain C (lane 2) using antisera prepared against *A. paragallinarum* strain A or against *A. paragallinarum* strain C

PCR amplification:

The *ha* gene was amplified form both tested strains (A ***and C)***. A very clear band with nearly the same intensity was observed migrating at ~ 1000 bp (Photo 2).

Gene sequencing:

The complete nucleotide sequence of the *ha* gene amplified from ***A. paragallinarum*** strain A and C were aligned using the CLC software - clustal *W*. As seen in Fig (3), there were 3 SNPs (single nucleotide polymorphism) at position 17(T/C), position 46 (G/A) and position 178 (T/C), and a single deletion was observed in strain C, at nucleotide position 94 – 102 which resulted in the absence of an alpha helix secondary structure area at amino acid position of 55 – 60 of the mature haemagglutinin protein antigen in the ***A. paragallinarum*** strain C.

Dot blotting analysis (fig 4) of ***A. paragallinarum*** strain A against strain C showed difference only in the first 100 bp with no repeats or inverted repeats observed along the whole gene sequence.

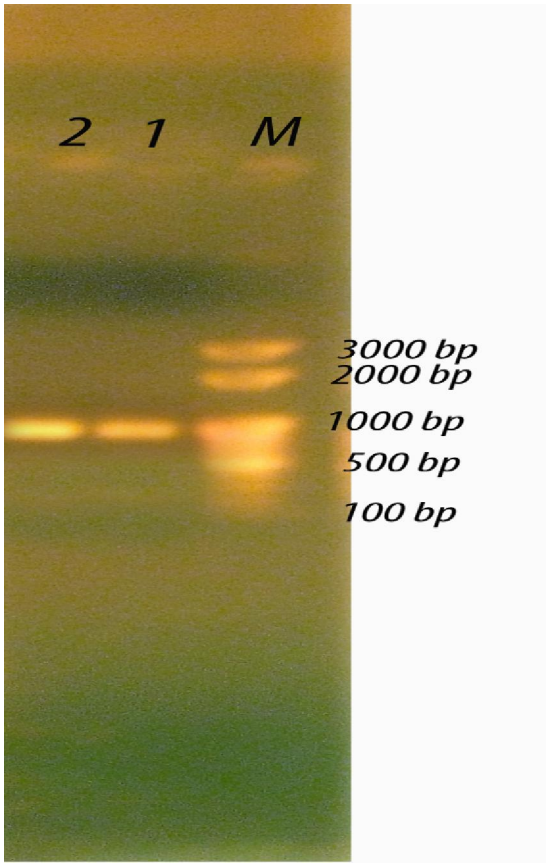
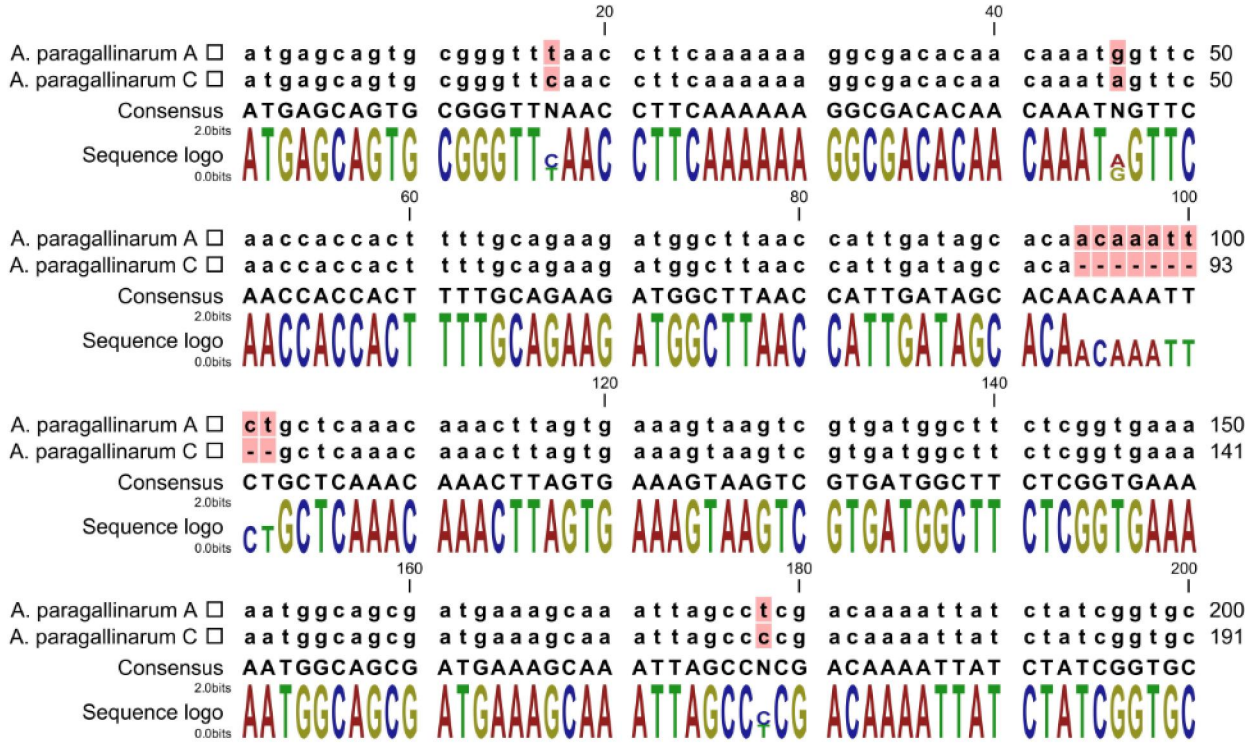
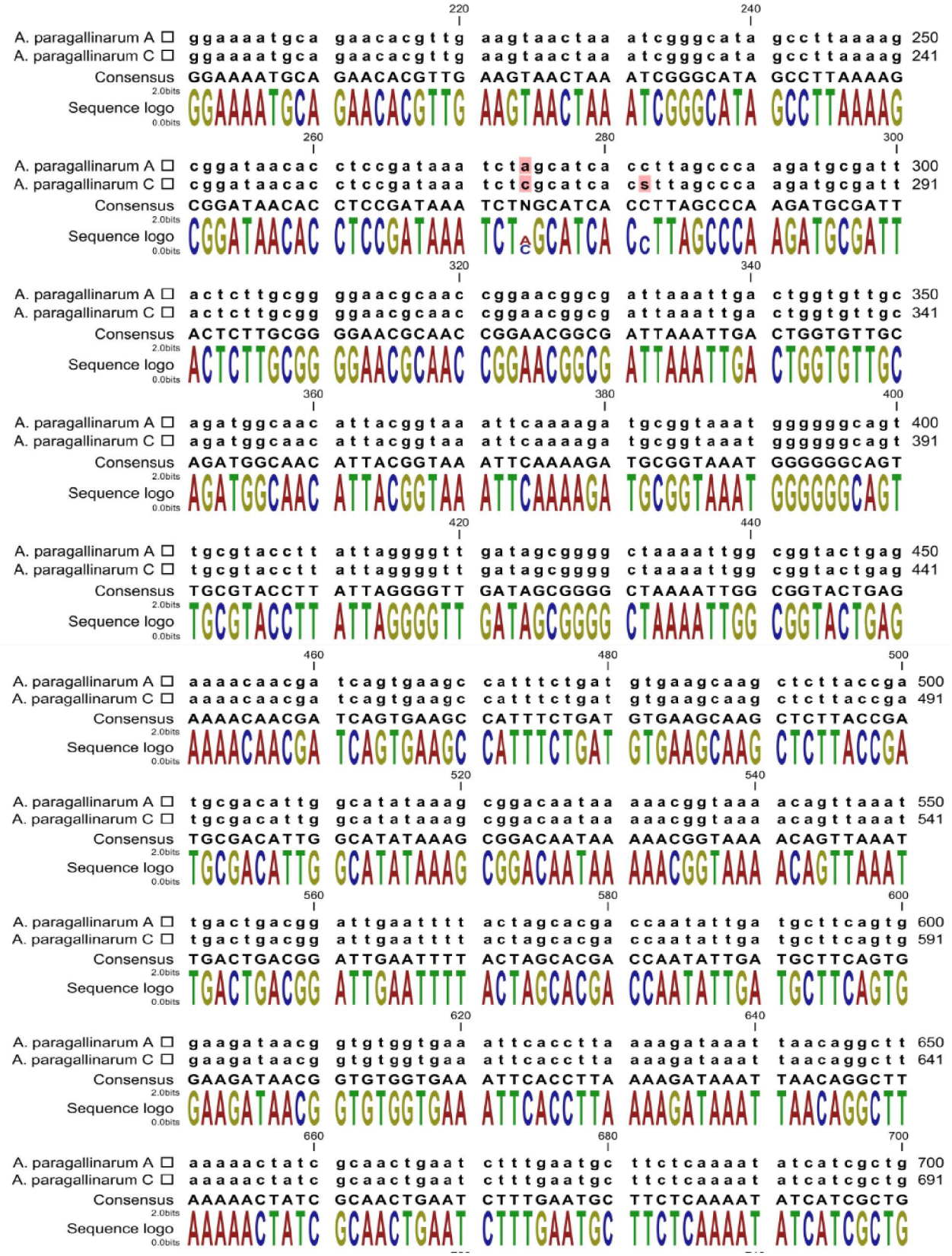


Photo (2) PCR results of amplification of *ha* gene from *A. paragallinarum strain A* (lane 1), and *C* (lane 2) note the clear band migrates about 1000 bp (M is 100 bp DNA ladder)




220
240
A. paragallinarum A ggaaaatgca gaacacgttg aagtaactaa atcgggcata gccttaaaag 250
A. paragallinarum C ggaaaatgca gaacacgttg aagtaactaa atcgggcata gccttaaaag 241
Consensus GGAAAATGCA GAACACGTTG AAGTAACTAA ATCGGGCATA GCCTTAAAAG
2.0bits
Sequence logo
0.0bits
260
280
300
A. paragallinarum A cggataacac ctccgataaa tctagcatca ccttagccca agatgcgatt 300
A. paragallinarum C cggataacac ctccgataaa tctcgcatca csttagccca agatgcgatt 291
Consensus CGGATAACAC CTCCGATAAA TCTNGCATCA CCTTAGCCCA AGATGCGATT
2.0bits
Sequence logo
0.0bits
320
340
A. paragallinarum A actcttgcgg ggaacgcaac cggaacggcg attaaattga ctggtgttgc 350
A. paragallinarum C actcttgcgg ggaacgcaac cggaacggcg attaaattga ctggtgttgc 341
Consensus ACTCTTGCGG GGAACGCAAC CGGAACGGCG ATTAAATTGA CTGGTGTTGC
2.0bits
Sequence logo
0.0bits
360
380
400
A. paragallinarum A agatggcaac attacggtaa attcaaaaga tgcggtaaat gggggggcagt 400
A. paragallinarum C agatggcaac attacggtaa attcaaaaga tgcggtaaat gggggggcagt 391
Consensus AGATGGCAAC ATTACGGTAA ATTCAAAAGA TGCGGTAAAT GGGGGGCAGT
2.0bits
Sequence logo
0.0bits
420
440
A. paragallinarum A tgcgtacctt attaggggtt gatagcgggg ctaaaattgg cggtactgag 450
A. paragallinarum C tgcgtacctt attaggggtt gatagcgggg ctaaaattgg cggtactgag 441
Consensus TGCGTACCTT ATTAGGGGTT GATAGCGGGG CTAAAATTGG CGGTACTGAG
2.0bits
Sequence logo
0.0bits
460
480
500
A. paragallinarum A aaaacaacga tcagtgaagc catttctgat gtgaagcaag ctcttaccga 500
A. paragallinarum C aaaacaacga tcagtgaagc catttctgat gtgaagcaag ctcttaccga 491
Consensus AAAACAACGA TCAGTGAAGC CATTTCTGAT GTGAAGCAAG CTCTTACCGA
2.0bits
Sequence logo
0.0bits
520
540
A. paragallinarum A tgcgacattg gcatataaag cggacaataa aaacggtaaa acagttaaat 550
A. paragallinarum C tgcgacattg gcatataaag cggacaataa aaacggtaaa acagttaaat 541
Consensus TGCGACATTG GCATATAAAG CGGACAATAA AAACGGTAAA ACAGTTAAAT
2.0bits
Sequence logo
0.0bits
560
580
600
A. paragallinarum A tgactgacgg attgaatttt actagcacga ccaatattga tgcttcagtg 600
A. paragallinarum C tgactgacgg attgaatttt actagcacga ccaatattga tgcttcagtg 591
Consensus TGACTGACGG ATTGAATTTT ACTAGCACGA CCAATATTGA TGCTTCAGTG
2.0bits
Sequence logo
0.0bits
620
640
A. paragallinarum A gaagataacg gtgtggtgaa attcacctta aaagataaat taacaggctt 650
A. paragallinarum C gaagataacg gtgtggtgaa attcacctta aaagataaat taacaggctt 641
Consensus GAAGATAACG GTGTGGTGAA ATTCACCTTA AAAGATAAAT TAACAGGCTT
2.0bits
Sequence logo
0.0bits
660
680
700
A. paragallinarum A aaaaactatc gcaactgaat ctttgaatgc ttctcaaaat atcatcgctg 700
A. paragallinarum C aaaaactatc gcaactgaat ctttgaatgc ttctcaaaat atcatcgctg 691
Consensus AAAAACTATC GCAACTGAAT CTTTGAATGC TTCTCAAAAT ATCATCGCTG
2.0bits
Sequence logo
0.0bits

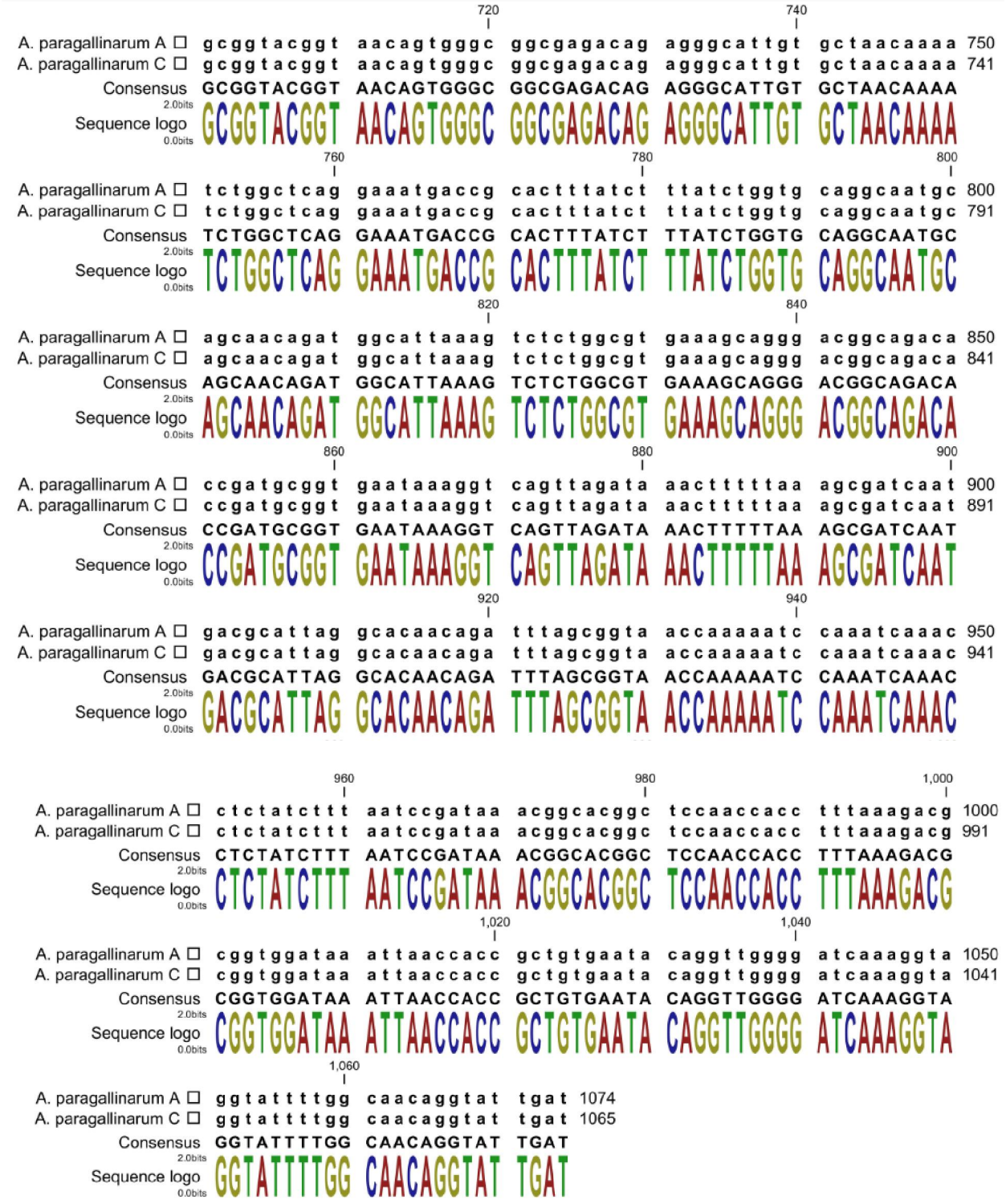


Fig (3) Sequence alignment of *haemagglutinin gene of A. paragallinarum* strain *A* and *C* showing the nucleotide orthologs (Sequence logo)

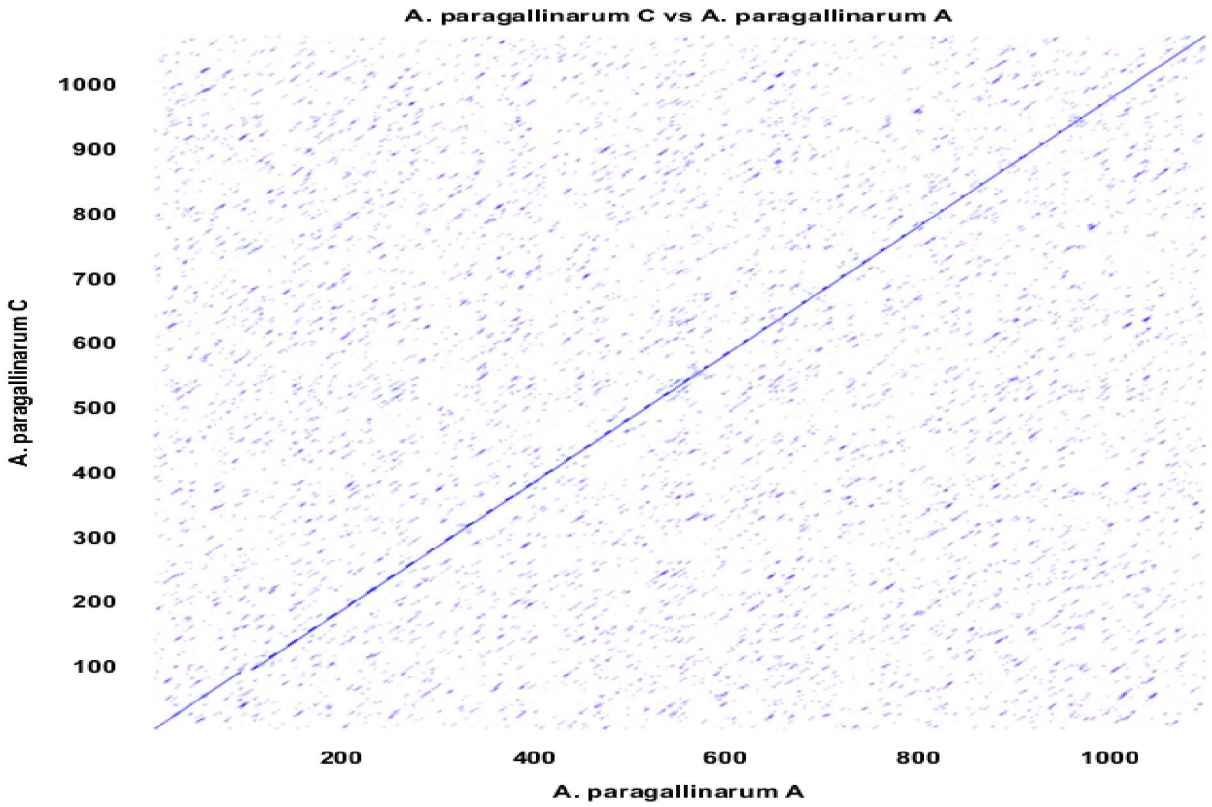
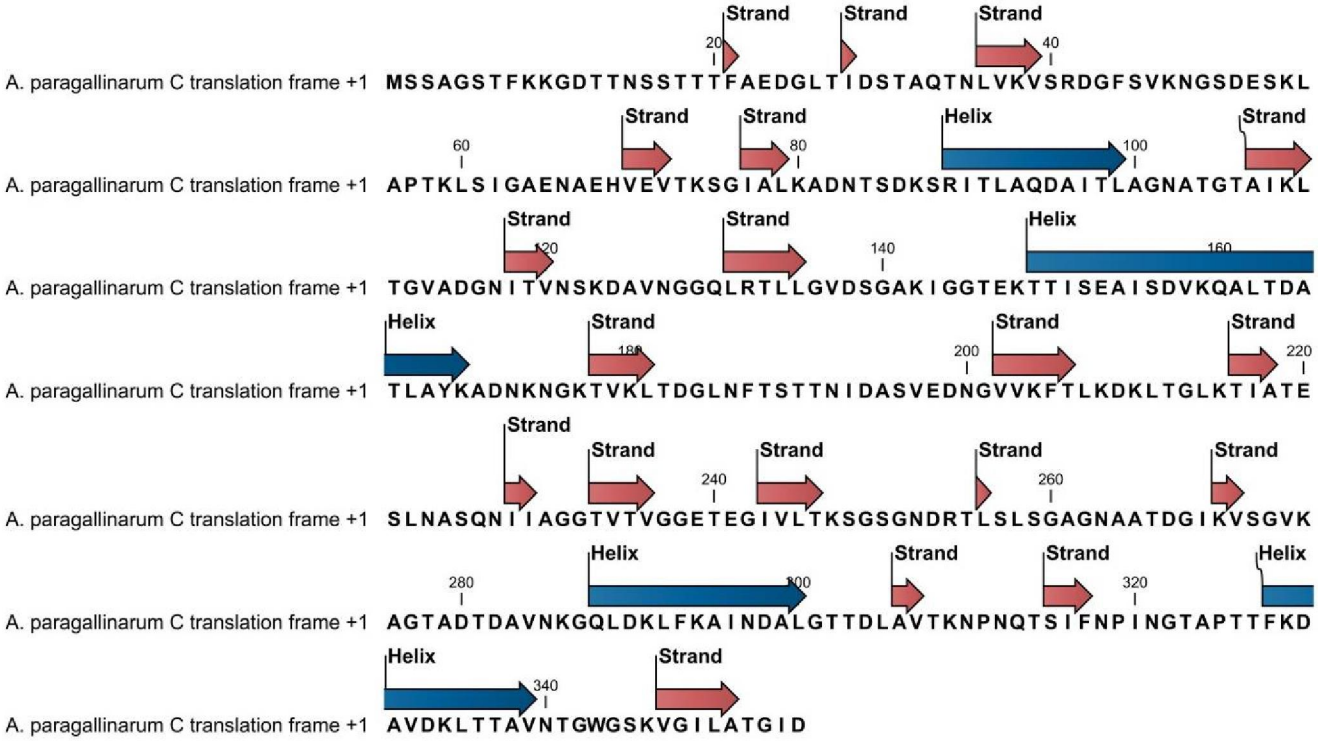


Fig (4) The Dot plot of the *ha* gene of *A. paragallinarum* strain A against strain C genes.



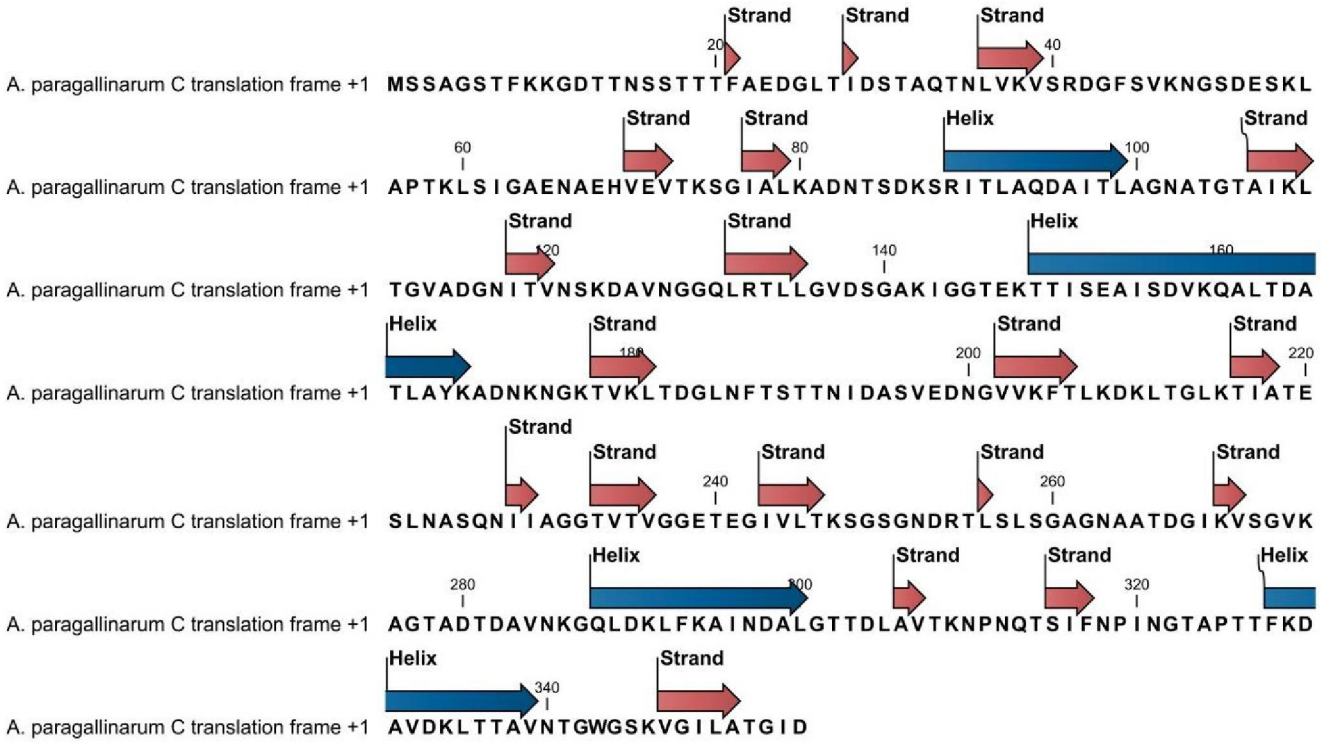


Fig (5) the secondary structure analysis of *haemagglutinin* protein of *A. paragallinarum* strain A and strain C. Note the absence of the α helix region in strain C at position 55 – 60.

4. Discussion

Infectious coryza control depends mainly upon vaccination .Commercial vaccines for infectious coryza, typically based on killed ***A. paragallinarum***, are widely available around the world (**Blackall, 1999**). Until recently, most of these vaccines contained only Page serovars A and C. This concept of a bivalent vaccine was based on the belief that Page serovar B was not a true serovar and that serovar A and C based vaccines provided cross-protection. However, because it has now been conclusively shown that Page serovar B is distinct, commercial trivalent vaccines are now available from the major international vaccine companies (**Jacobs et al., 1992**).

The traditional definitive method for the identification of ***A. paragallinarum*** requires the growth of the bacterium on NAD-dependent media and then an extensive biochemical characterization to confirm the identity of the strain (**Miflin et al., 1995**). This is a challenging set of requirements. ***A. paragallinarum*** is a fastidious, slow-growing organism. Hence, it is often overgrown by other, faster-growing commensals. Biochemical characterization requires the availability of specialized, expensive media that can support the growth of NAD-dependent bacteria. Then the application of HA test that could differentiate between the serovar A from C. Molecular approaches , on the other hand, could give a definitive tool to accurately identify the serovars and give deep insight view on the exact difference present between these serovars that used in the quality control of the vaccines.

A. paragallinarum strain A and C gave the same biochemical reactions. But on the proteomic level however, Protein profile of the 2 serovars gave some differences in band intensities on SDS-PAGE and western analysis. As seen in Fig 2, the bands between 40-55 KDa reacted with different intensities to sera against either strain, this is may be due to different expression level of the *ha* gene in both serovars used or due to the genetic differences which observed in the sequence analysis. The deletion mutation seen in serovar C at position 94-102, resulted in absence of an alpha helix region in the mature protein which might cause the antibodies reacted at this epitope become absent in the anti serovar C antibodies.

The genetic analysis of the haemagglutinin gene of both serovars indicating sharp differences in the form of three SNPs and one deletion. As mentioned before, the deletion resulted in absence of alpha helix region this area thus will be determinant

for serovar C and may be used as a method of identification of the serovar. The three SNPs observed resulted in changes in the amino acid coded in both A and C serovars (L/S , G/S and G/P). These point mutations could also be a molecular marker to distinguish the serovars. Such limited degree of variation was investigated by **Rhonda et al., 2002** during their sequencing analysis of 11 serotyping reference strains.

As a conclusion, molecular analysis of ***A. paragallinarum*** could accurately differentiate between serovar A and C on the genetic level due to the presence of three SNPs and one area of deletion. On proteomic level however, only differences in the level of expression in SDS-PAGE analysis and /or reactivity in western blotting analysis could be seen.

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