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Identity of *Pasteurella multocida* and Avian Influenza H9N2 Strains Used in Preparation of a Combined Inactivated Vaccine Using PCR

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Abstract: In this study, the local *Pasteurella multocida* and AI virus strains were identified using PCR. The genomic DNAs of *Pasteurella multocida* strains (1, 3, 4 and D2) were extracted and the estimated size was more than 23 kbp. PCR was applied on the extracted DNA using the specific primers of the *ompH* gene which successfully amplified giving PCR product at 1000bp. On the other hand, the RNA component of AIV serotype H9N2 was extracted and RT-PCR was processed where the amplified H9 gene showed characteristic band of 808 bp. In conclusion, such strains of *P. multocida* and AI were confirmed to be ready for the use in the preparation of targeted polyvalent inactivated vaccine for the protection of poultry against fowl cholera and H9N2 diseases.

Key words: Pasteurella multocida · AI · Identity · PCR · Vaccine

INTRODUCTION

P. multocida belongs to the family *Pasteurellaceae and* classified into five groups based on capsular antigens and into 16 serotypes based on LPS antigens [1]. *Pasteurella multocida* is a widespread Gram-negative eubacterium, alone or in association with other pathogens, is responsible for severe diseases in mammals (Including man), fowl cholera in poultry [2], hemorrhagic septicemia and shipping fever in cattle and atrophic rhinitis in pigs [3].

Fowl cholera, which is generally caused by serotypes A: 1, A: 3 or A: 4 is a highly contagious disease causing devasting economic losses to the poultry industry through deaths, weight loss and condemnation of carcasses worldwide [2]. Fowl Cholera occurs sporadically or enzootically as peracute, acute or chronic form all over the world [4]. Mortality may range from only few percent to nearly 100% and recovered birds may remain as carriers even after 9 weeks after infection [2]. OMP (Major Outer Membrane Protein) of *P. multocida* has been characterized as a porin. Porins are strong immunogens and have been demonstrated to be able to induce protective immunity in animals [5].

Avian influenza (AI) is an infectious respiratory disease of birds caused by influenza A viruses that are members of the family Orthomyxoviridae [6]. Influenza viruses type A are enveloped negative-sense, segmented, single-stranded RNA viruses and classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens [7]. At present, 16 H subtypes (H1-H16) and 9 N subtypes (N1–N9) are recognized. The H9N2 avian influenza virus (AIV) was reported to be of low pathogenicity in chickens [8, 9] causing minimal clinical signs other than a slight drop in egg production [10].

The aim of this study is to molecularly identify the microbial organisms that will be used in the preparation of a combined inactivated bivalent vaccine for the protection of chickens against fowl cholera and avian influenza H9N2 diseases using PCR.

MATERIALS AND METHODS.

Isolation of *P. multocida* DNA: The genomic DNAs of *Pasteurella multocida* serotypes (1, 3, 4 and D2) that obtained from the Strain Bank, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbassia, Cairo, Were extracted using Isolate Genomic DNA Mini Kit.

Polymerase Chain Reaction:

Oligonucleotide Primers for Detection of *P. multocida*: The primers sequences were chosen for *ompH* gene [11]:

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Forward primer: 5- ACTATGAAAAAGACAATCGTAG-3
Reverse primer: 5-GATCCATTCCTTGCAACATATT-3

Amplification of *OmpH* Gene: The isolated genomic DNAs were amplified using PCR according to according to Luo *et al.* [11] as follow. PCR mixture consisted of 5 µl of *P. multocida* genomic DNA, 1 µl of Taq DNA polymerase (I U/ µl), 60 pmol of primer 1 and primer 2 each, 1.5 mM $MgCl_2$ and 0.1 mM dNTPs in 50 µl double distilled water. The amplification reactions were performed under following conditions: 94°C for 5 min, then 35 cycles each at 94°C for 15 sec, 55°C for 1 min and 72°C for 1 min.; lastly 72°C for 10 min. The PCR products were analyzed on 1% agarose gel.

Viral RNA Isolation and RT-PCR [12]: Viral RNA of AI H9N2 that isolated in the Central Laboratory for Evaluation of Veterinary Biologics, Abbassia, Cairo, was directly extracted from allantoic fluid of the inoculated SPF-ECE using QIA amp viral RNA Mini-Qiagen RNA extraction kit. The RNA was transcribed into cDNA then H9 amplified by its specific primers: Forward primer: AGCAAAAGCAGGGGAAYWWC, Reverse primer: CCATACCATGGGGCAATTAG according to Siddique *et al.* [12]. The reaction mixture contained AIV H9 specific primers along with other reagents from the Kit were run in a thermal cycler using the following thermal parameters:

Reverse transcription at 42°C for 55min followed by 94°C for 2min, then 30 cycles of 94°C for 15sec, 50°C for 10-20sec and 68°C for 2min. lastly 72°C for 2min. The amplified and kb DNA marker were run on 1% Agarose.

RESULTS AND DISCUSSION

Fowl cholera [2] and A.I. [13] are two of the major economically important respiratory and septicaemic disease of poultry in Egypt and allover the world. They are highly contagious diseases causing devasting economic losses to the poultry industry through death, weight loss and condemnation of carcasses worldwide.

Identity and confirmatory processes should be applied on the master seed of any vaccine prior to the preparation of the target vaccine. A combined vaccine against fowl cholera and A.I. are the target of the second part of this study.

In the present study, the local *P. multocida* and A.I. strains that will be used in the preparation of combined inactivated vaccine against fowl cholera and AI were identified using PCR. One of the key steps in the PCR is the identification of the gene(s) to be amplified.

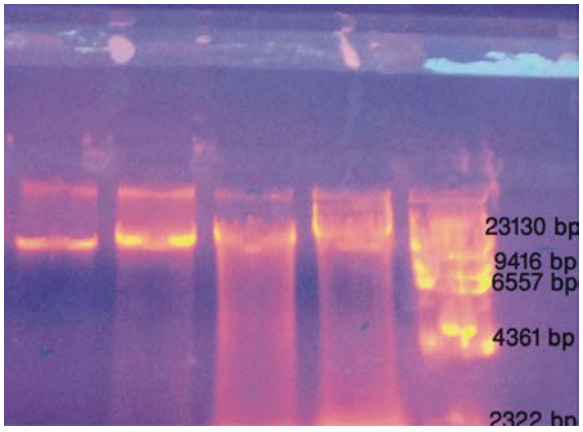

Fig. 1: Shows the extracted genomic DNA of *P. multocida* strains

OmpH is a protein involves in the synthesis of LPS [14] or in the exportation of macromolecules through the bacterial envelope as recently demonstrated [15]. Protein H or porin H is the major outer membrane protein in the envelope of *Pasteurella multocida* [16].

Firstly the genomic DNAs of *Pasteurella multocida* strains (1,3, 4 and D2) were extracted, fractionated on 0.7% agarose and the estimated size of the genomic DNA of different *Pasteurella multocida* strains was more than 23 kbp as shown in photo (1). The same size also was obtained by AFAF [17] who extracted the genomic DNA from *Pasteurella multocida* strains (CU, 1, 3, 4 and D2) using traditional phenol chloroform method and reported that the estimated size of the genomic DNA of different *Pasteurella multocida* strains was more than 23 kbp.

The *ompH* gene of the examined strains was successfully amplified with its specific primers. The PCR products of the examined strains appeared under the U.V transluminator at 1000bp as shown in Photo (2). The same results were obtained by Luo *et al.* [11] who recorded the same PCR products from *Pasteurella multocida* strains (CU, 1, 3 and 4). In this concern, they recorded that the *ompH* gene is conserved among all of the *Pasteurella multocida* serotypes.

For AIV serotype H9N2 the RNA was directly extracted from allantoic fluid of the inoculated SPF-ECE for conducting RT- PCR then transcribed into cDNA then H9 gene was amplified. The gel electrophoresis of the amplified H9 gene showed characteristic band of 808 base pair as shown in photo (3). The same result was obtained by Siddique *et al*. [12].

From the previously mentioned results, the under testing bacterial and viral strains were identified using the PCR assay and such strains were confirmed to be ready

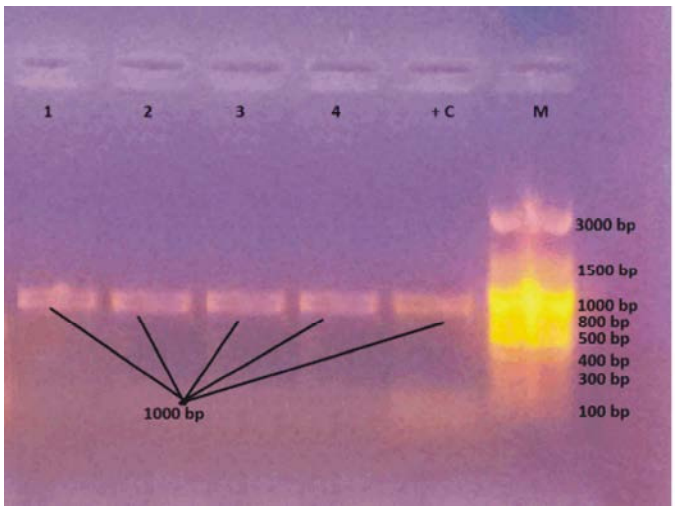

Fig. 2: Shows the result of PCR product of *ompH* gene from whole genomic DNA of *P. multocida* strains.
M, marker; Lane 1, *P. multocida* serotype1; Lane 2, *P. multocida* serotype3; Lane 3, *P. multocida* serotype 4; Lane 4, *P. multocida* serotypeD2; +C, positive *omph* gene control

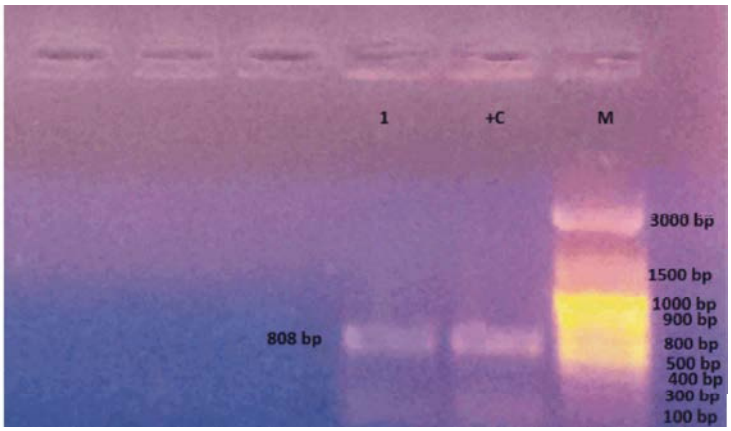

Fig. 3: PCR product of H9 gene of A.I. virus.
M, marker; Lane 1, AI H9N2; +C, positive H9 control

for the use in the preparation of targeted polyvalent inactivated vaccine for the protection of poultry against fowl cholera and H9N2 diseases.

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