



Short Communication

Retrospective Detection of *Avibacterium Paragallinarum* Serovar B in Egg Yolk Materials by PCR

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ABSTRACT

Avibacterium paragallinarum is Gram-negative bacteria that cause infectious coryza (IC), an acute respiratory disease of chickens. Despite vaccination of Layer and breeder farms of Iran against IC, they are still experiencing the disease clinically and there is no knowledge of serotypes prevalence of this bacterium in this country. This study designed to determine serovar identity by molecular identification tests of previously studied isolates from Iran that were inactivated accidentally in egg yolk storing culture media. Genomes of the isolates extracted from egg yolk and then were subjected to two set of PCR tests using by published group specific and Page's serovar specific primers. The later primers were based on sequence differences in hypervariable region of haemagglutinin protein of *Av. paragallinarum*. These two sequential PCR confirmed that the bacteria were *Av. paragallinarum* and were associated to Page's serotype B. This is the first report of molecular detection of serotype B of *Av. paragallinarum* from Iran.

Keywords: *Avibacterium paragallinarum*, molecular identification, egg yolk, Serotyping, Infectious Coryza

INTRODUCTION

The *Avibacterium paragallinarum* is a Gram-negative bacterium (formerly *Haemophilus paragallinarum*) cause infectious Coryza (IC), an acute respiratory disease of chickens (Blackall 2008). IC occurs in all countries with chicken rising industries and imposes economic losses including increased cull birds and egg production loss from 10% to 40% (Blackall 1999). IC up to the past ten years is diagnosed by isolation and identification of *Av. paragallinarum* based on its biochemical properties (Blackall & Matsumoto 2003). *Av. paragallinarum* generally

requires NADH (Reduced nicotinamide adenine dinucleotide) as a growth factor (page 1962). For this reason, isolation requires co-cultivation with *Staphylococcus epidermidis* (page 1962) or *Staphylococcus hyicus* as a feeder (Blackall & Reid 1982) to supply this requirement. *Av. paragallinarum* is also a relatively slow growing organism, which produces tiny dewdrop colonies and can be overgrown by other bacteria in diagnostic samples (Chen *et al* 1998). Furthermore, biochemical characterization of isolates requires the use of expensive specialized media (Blackall 1999). Overall isolation and phenotypic characterization require several days to complete (Blackall 1999). Nucleic acid molecular tests for diagnosis and identification of *Av. paragallinarum*

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have been developed and evaluated by some researchers (Bisgaard *et al* 2012, Chen *et al* 1998, Chen *et al* 1996, Morales-Erasto *et al* 2011, Sakamoto *et al* 2012). These tests could be comparable with culture and haemagglutination inhibition (HI) serotyping methods. HPG-2 PCR even reported to be a reliable to identify NAD-independent *Haemophilus paragallinarum* (Miflin *et al* 1999). There is no report about serovar detection of *Av. paragallinarum* in Iran. We used here PCR test to detect the bacteria and determine serovar identity of previously isolated *Av. paragallinarum* which were stored in egg yolk culture media and became inactivated for temperature changes.

MATERIALS AND METHODS

Bacteria. Two isolates of bacteria of *Av. paragallinarum* that have previously been characterized biochemically by Banani (2007) were selected for molecular study. They had been isolated from layer flocks of khorasan province of Iran in 2001 with no history of vaccination against IC. These field isolates preserved in egg yolk at -70 °C and has been inactivated accidentally because of refrigerator failure.

Bacterial DNA extraction. DNA extractions were essentially performed by phenol–chloroform method as described by David Moore (2002), with minor modifications. Briefly, 400 µl of yolk containing *Av. paragallinarum* were collected to 1.5µl microtubes after thawing at room temperature. Repeating freezing and re-thawing has been done one more time again at -20 °C and 45°C respectively. Uninfected egg yolk also considered as negative control. After centrifugation at 7,000 rpm for 5 min the supernatants were removed (100µl). The remainder was mixed and shaken in 300 µl of STE buffer (50 mM Tris, 50 mM ethylene diamine tetra acetic acid [EDTA], 100 mM NaCl, 1% (w/v) sodium dodecyl sulfate[SDS], and proteinase K (5mg/ml) and incubated at 56 °C for 4 hr with two times inverting every hour. Equal volumes of phenol and chloroform were used for purification. DNA was precipitated with 3 M sodium acetate and an equal volume of ethanol. The precipitated nuclei acid was

washed with 70% ethanol and finally re-suspended with distilled water. Purity and concentration of extracted DNA was estimated by Nanodrop® (ND_1000) spectrophotometer. DNA extract of only one available isolate (serotype A) in our Lab and *Ornithobacterium rhinotracheale* that were previously characterized was used as positive and negative bacterial control genome respectively.

***Av. paragallinarum* identification.** extracted DNAs were tested using HPG-2 PCR test as described by Chen *et al.* (1996) which known to be specific for *Av. paragallinarum* (Blackall, 2008). The primers set that introduced with this test was designated as N1 and R1 (table 1). These primers expected to generates an amplicon of approximately 500bp and up to now there is no report of primers location in target sequences of *Av. paragallinarum* genome. This PCR was carried out in 25-µl volumes. The reaction prepared using AccuPower® PreMix. The appropriate amount of template DNA (15-150 ng) was added. The MgCl₂ and primer concentrations were determined by an optimization experiment. Amplification was performed by an Eppendorf® (Mastercycler) thermal cycler in which the reaction condition were programmed to denaturation at 95 °C for 5 min then 30 cycles of 94 °C for 30sec, 58 °C for 45sec, and 72 °C for 45sec followed by a final extension cycle of 72 °C for 5 min. Finally PCR products were detected by 1% agarose gel electrophoresis for 25 min at 80V and visualization under UV light (Figure 1).

Table 1. primers used to identification of *Av. Paragallinarum*

Primer	Sequences
R1	5'-CAA GGT ATC GAT CGT CTC TCT ACT-3'
N1	5'-TGA GGG TAG TCT TGC ACG CGA AT-3'

***Av. paragallinarum* serotyping.** To determine the association of isolates to which type of *Av. Paragallinarum* Page's serovars, (A, B, and C), a multiplex PCR test that previously conducted by Sakamoto *et al.* (2012) was considered. Primer sets which were used are based on hypervariable sequences of outer membrane haemagglutinin protein gene of bacterial cell wall (Table 2). The reaction mixture has

been prepared using AccuPower® Premix according to pervious experiment but in 50µl volumes. Amplification was achieved with an initial denaturation step at 98 °C for 7 min and 30 cycles of 98 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min; and a final step at 72 °C for 7 min. The PCR products were analyzed by agarose electrophoresis in a manner of previous experiment. For further confirmation of multiplex PCR results, two PCR also has been run by each primers set separately in identical condition of multiplex PCR.

Table 2. primers used to serotyping of *Av. Paragallinarum* (Sakamoto et al 2012)

Primer	Sequence	Fragment length
Common forward	5'-GGCTCACAGCTTTATGCAACGAA-3'	
A reverse	5'-CGCGGGATTGTTGATTTTGT-3'	0.8 kbp
B reverse	5'-GGTGAATTCACCACACCAC-3'	1.1 kbp
C reverse	5'-TAATTTTCTTATCCAGCATCAATACCAT-3'	1.6 kbp

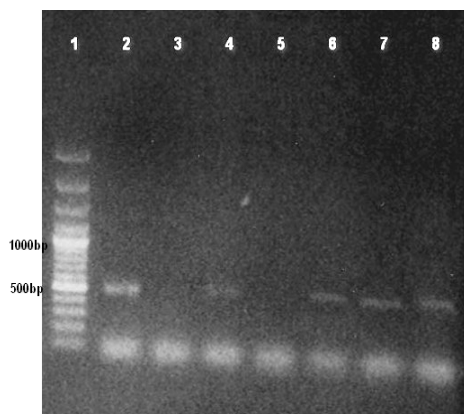


Figure 1. PCR product electrophoresis of *Av. Paragallinarum* identification in different egg yolk stock sample of containing inactivated bacteria. 1, 100bp Ladder DNA. 2, positive control. 3, uninfected egg yolk. 4 and 6-8, different extracts of infected egg yolk. 5, DNA extract of ORT.

RESULTS AND DISCUSSION

According to the gel electrophoresis the sizes of HPD-2 PCR amplicons were approximately 500 bp (Figure 1). The exact HPD-2 PCR product in identifying the *Av. paragallinarum* is 516 bp according to Miflin et al. (1999). Definitive diagnosis of IC would be difficult in flocks, where there is mixed infection with other conditions and/or pathogens such as ORT,

fowl cholera, chronic respiratory disease, chronic fowl cholera, fowl pox, swollen head syndrome, A-avitaminosis, (Blackall 2008) and *Pseudomonas aeruginosa* (Giurov 1984) which represent similar clinical signs. Isolation and identification of *Av. paragallinarum* by bacteriological and serological methods in spite of some complexity and experience demanding (Chen et al 1996) have so far been the main methods of identification. HP-2 PCR in IC diagnosis and research on *Av. paragallinarum* species have been used by some researchers (Byarugaba et al 2007, Chen et al 1998, Christensen et al 2003, Chukiatsiri et al 2012). Nucleic acid based molecular tests turned out be promising, alternative and reliable test for IC related researches. Although HP-2 PCR was developed by a random cloning method of bacterial genome, the role and locus of the target gene is not known yet. But multiplex PCR test introduced by Sakamoto et al, (2012), used for serotyping, targeted the hypervariable region of HMTp210 sequence, which encodes the HA antigen of *Av. Paragallinarum*. This test also used successfully by Chukiatsiri et al. (2012) in studies of IC cases. In this study by using Multiplex PCR test and also another separated PCR for serotyping (A, B, C) on DNA extracts of infected egg yolk resulted in approximately 1.1 bp products in both of the examined cases (Figure 2).

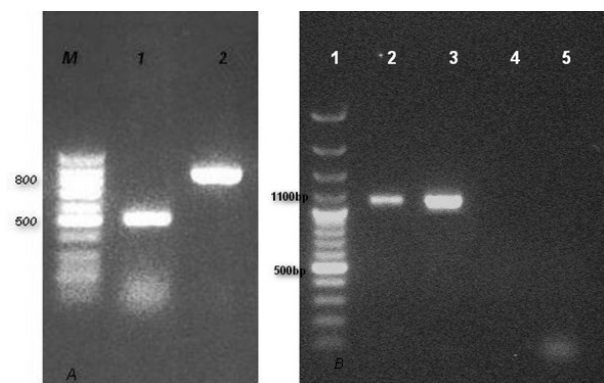


Figure 2. Agarose gel electrophoresis of PCR product to serotyping of *Av. paragallinarum*. (A) serovar A serves as positive control: M, 100bp lader. 1, group specific band. 2, serovar specific band of A. (B) chicken egg yolk PCR electrophoresis :1, DNA 100bp lader. 2 and 3, two DNA extract of infected egg yolk. 4, blank well. 5, DNA extract of ORT.

According to this size, it was identified as serovar B in Page's scheme of classification of *Av. paragallinarum*. In all tests negative controls including uninfected egg yolk and ORT samples did not show any amplification. Detection of serovar B was confirmed by detection of two different locations of bacterial genomes, first using HP-2 PCR on non haemagglutinin related genome and second haemagglutinin protein genome which has important role in pathogenesis and immune-protection of microorganism (Barnard *et al* 2008, Wu *et al* 2011). IC clinically has been diagnosed in layer and breeder flocks of Iran and vaccination program against it have been recommended by government authorities. *Av. paragallinarum* isolation and tests for its susceptibility against antibiotics has been done in Iran (Banani 2007). But there is no documented report of serological or molecular serovar studies of the IC agents around the country. We decided in our laboratory for the first step to have retrospective study of molecular serotyping on two available inactivated isolates which were preserved in chicken egg yolk. After obtaining negative results by some commercial DNA extraction kits, DNA extraction of bacterial genome from egg yolk using phenol-chloroform method was preferred and optimized. The authors could not find any information about bacterial DNA extraction from chicken egg yolk for PCR. Extraction of pure chicken egg yolk DNA previously has been described by SUN Lijun (1998). He did Ficoll-400 density gradient centrifugation prior DNA extraction. Phenol-chloroform DNA extraction procedure in spite of having health concerns for laboratory stuffs, inevitably could have advantageous to use in retrospective studies of inactivated or active *Av. paragallinarum* preserved in chicken egg yolk, which is one of the recommended medium used to long term preservation of the *Av. paragallinarum* (Blackall 2008). Serovar B previously reported from some countries in Asia, America, Africa and Europe continents (Blackall 2008). This is the first detection of Page's serovar B of *Av. paragallinarum* from Iran. These isolates in an experimental infection of 6 weeks

old SPF chicken by intrasinus inoculation induced facial swelling, sever periorbital sinus inflammation (Banani 2007), Although there have been some report that serovar B shows low pathogenicity in comparison to other serovars of *Av. paragallinarum* (Soriano *et al* 2004), but the pathogenicity of *Av. paragallinarum* can vary according to factors such as the growth conditions, dose of bacteria, passage history of the isolate and the state of the host (Blackall, 2008). In our country vaccination against IC is performed by bacterin vaccine containing of serovar A, B and C. It seems that detection of apparently only serovar B in both flock would be interesting and need more attention as there is evidence that the cross-protection within Page's serovar B strains is partial (Byarugaba *et al* 2007, Jacobs *et al* 2003). General recommendation for IC prevention is preparation of an autogenous bacterin of Page's serovar B along side of standard serovar of A, B, and C (Jacobs *et al* 2003). We believe more studies are needed to be considered for illustration of seroprevalence of *Av. Paragallinarum* in Iran. Unfortunately in our studies we used all of bacterial samples in optimization of DNA extraction and couldn't be able to reach all of our research goals, such as sequencing of PCR products and BLASTing with available gene bank databases.

Ethics

Hereby, I declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors have no conflict of interest.

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