ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 5 (7): 623-626, 2006
ISSN 1682-8356

A Simple Protocol for Amplification of Genes from Inactivated Oil Adjuvant Fowl Cholera Vaccine

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Abstract: Pasteurellosis is one of the most contagious disease of avians. Control of pasteurellosis is at present carried out by vaccination with whole-cell bacterins, which confer serotype specific protection. Outermembrane proteins (OMP) of Gram negative bacteria are considered the most important immunogens and have been proved to confer immunity. A simple protocol for amplification of outermembrane protein genes from inactivated bacterial vaccines has been standardized. An attempt has been made to compare the OMP genes of field isolates and vaccine strain.

Key words: *Pasteurella multocida*, OMPH gene, Vaccine strain, Restriction enzyme analysis

Introduction

Avian pasteurellosis is a contagious septicaemic disease associated with high morbidity and mortality and remains a major concern to the poultry industry despite 100 years of research. Control of pasteurellosis is at present carried out by vaccination with whole-cell bacterins, which confer serotype specific protection.

Several studies have been carried out to identify the important immunogens of *Pasteurella multocida*. Lu *et al*. (1991) demonstrated that vaccination with outer membrane proteins (OMP) protected rabbits against homologous challenge. OmpH specific monoclonal antibodies inhibited the proliferation of *P. multocida* in lungs of mice (Marandi and Mittal, 1997). Hence in the present study an attempt was made to amplify the OmpH gene of *P. multocida* isolated from ducks in Kerala and compare the same with those present in the vaccine used. Since no protocol was available for amplification of genes from inactivated bacterial vaccines a simple protocol was devised.

Materials and Methods

Pasteurella strains: Twenty-five isolates (DP1-DP25) of *P. multocida* obtained from ducks from different regions of Kerala, INDIA were used in the study. A reference isolate of *P.multocida* from fowl (LKO) obtained from Indian Veterinary Research Institute, Izatnagar, INDIA was used for comparison. All the isolates were identified as *P.multocida* based on morphology, cultural characters and biochemical reactions as described by Barrow and Feltham (1993).

Cholerin Triple – Fowl Cholera bacterin, inactivated oil adjuvant vaccine containing *P. multocida* serotypes A:1, A:3 and A: 4. Manufactured by ABIC, Israel and marketed by M/s Sarabhai Chemicals, Baroda, was used in the study.

Preparation of template DNA from *P. multocida* cultures Polymerase Chain Reaction was conducted using bacterial culture lysates as template DNA. A pure colony of *P. multocida* was inoculated into five millilitres of Brain Heart Iinfusion broth and incubated at 37°C for 18 h. One point five millilitres of this broth culture was transferred to an Eppendorf tube and centrifuged at 3000 x g for 10 min. The pellet was washed twice in PBS and the final pellet was resuspended in 100 µl of triple distilled water. The mixture was boiled for 10 min and immediately chilled on ice for 30 min. The sample was then thawed and centrifuged at 3000 x g for 5 min. The supernatant was stored at -20°C for further use as template DNA.

Preparation of template DNA from Cholerin Triple vaccine: Two millilitres of Cholerin Triple vaccine was mixed with eight milliliters of isopropanol in a test-tube and vortexed for five minutes. The mixture was then centrifuged at 3000 X g for 10 min. The oil phase on the top and the aqueous phase of the vaccine below, which was mixed with isopropanol were pipetted out. The pellet containing the bacterin was washed twice in PBS, pH 7.2. The PBS was decanted and the washed pellet was resuspended in 100 il of triple distilled water and boiled at 100°C for 10 min. The mixture was then frozen at -20°C for 30 min, thawed and centrifuged at 3000 x g for 10 min. The supernatant was used as template DNA for PCR.

Oligonucleotide primers: Two oligonucleotides based on the sequence of *P. multocida* X-73 *ompH* gene, Accession No. U50907 (Luo *et al*., 1997) were designed using Primer3 software. The primers were custom synthesized by M/s Bangalore Genei India. The sequences of the two primers were as follows:

OmpH 1:5'-GCG TTT CAT TCA AAG CAT CTC-3'-21 mer
and
OmpH 2: 5'-ATG ACC GCG TAA CGA CTT TC -3'-20 mer

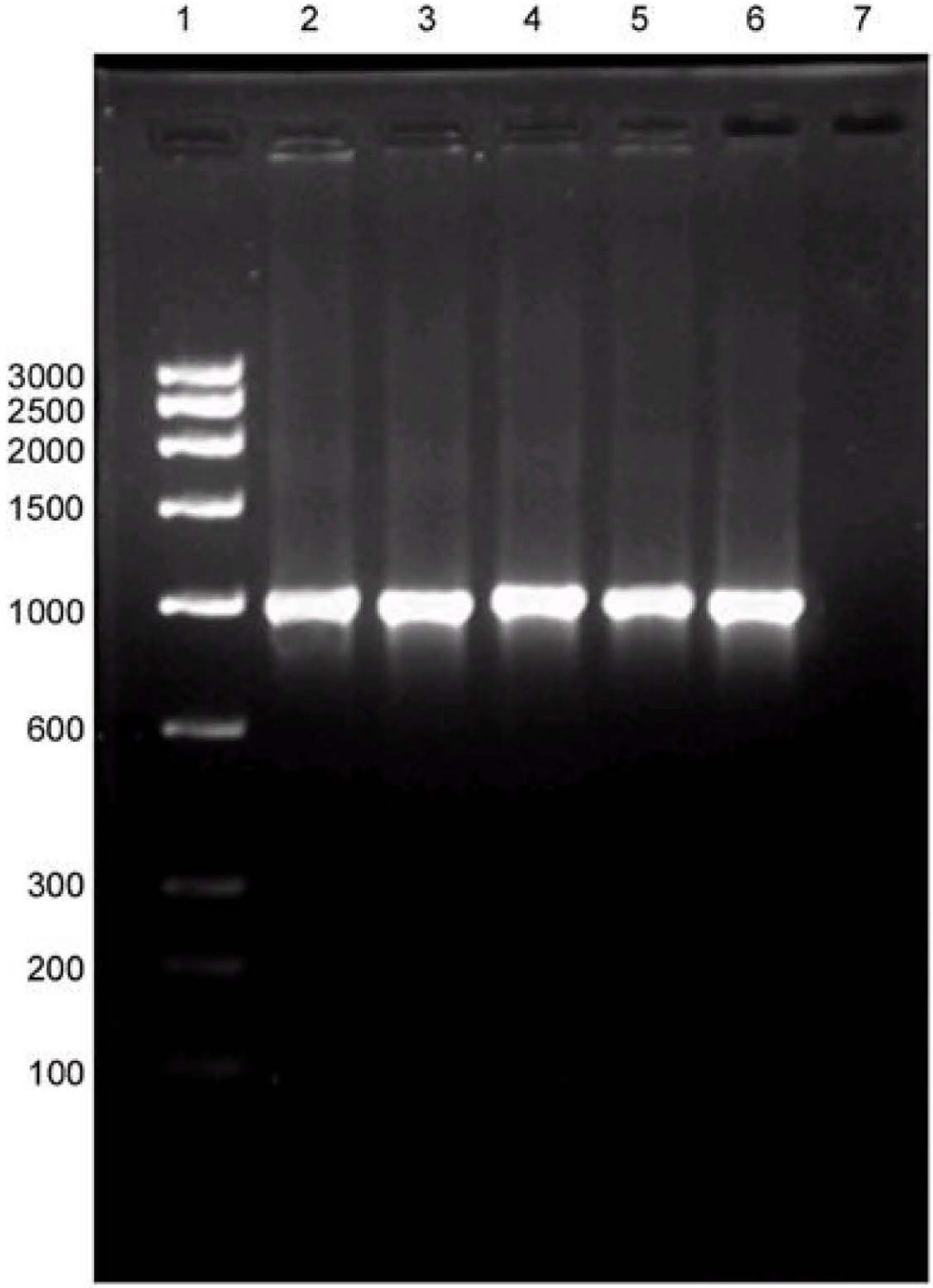

Fig. 1: Amplification of OmpH gene of *P. Multocida*
Lane 1 Low range DNA marker
Lane 2-5 LKO, DP 1, DP8 & DP21
Lane 6 Vaccine
Lane 7 Negative control

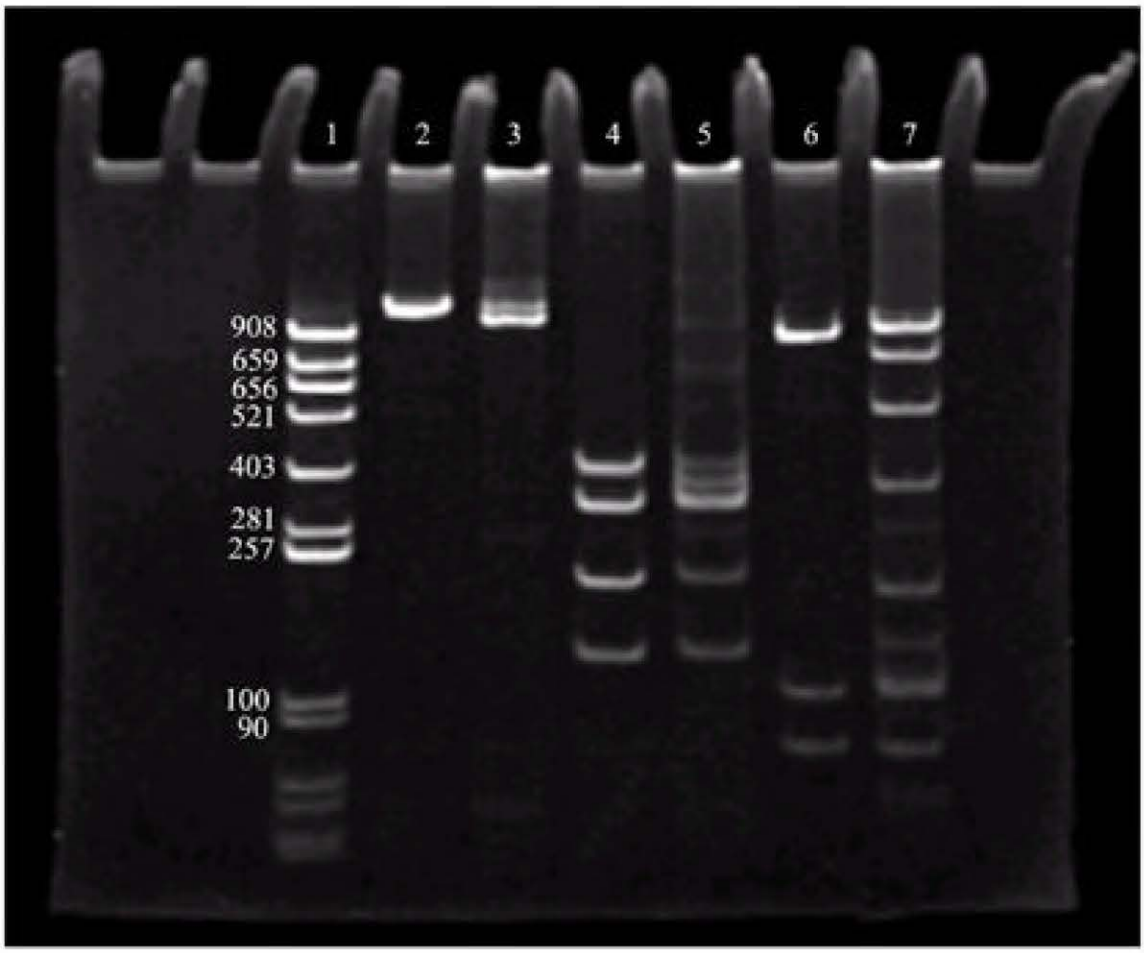

Fig. 2: REA of OmpH-PCR products from inactivated FC vaccine
Lane 1: pBR 322 DNA/*Alu* I digest marker
Lane 2: Undigested OmpH-PCR product from DP 1
Lane 3: Undigested OmpH-PCR products from vaccine
Lane 4: OmpH-PCR product of DP1 digested with *Dra* I
Lane 5: Omph-PCR products of vaccine digested with *Dra* I
Lane 6: OmpH-PCR product of DP I digested with *Hinf* I
Lane 7: OmpH-PCR products of vaccine digested with *Hinf* I

Amplification of ompH gene

PCR conditions: A 50 µl reaction mixture was prepared in 0.2 ml thin walled PCR tube. Five microlitres of template DNA was added to a reaction mixture containing 40 pmoles of primer OmpH 1 and primer OmpH 2 each, 200 µ M of each dNTP, 1 X *Taq* buffer with 1.5 M $MgCl_2$ and 2 units of *Taq* DNA polymerase. The amplification reaction was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme, an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The template DNA used was bacterial culture lysate. The product was analysed by two per cent submarine agarose gel electrophoresis. Standard molecular size marker low range DNA ruler with fragments 3000, 2500, 2000, 1500, 1000, 600, 300 and 100 bp was used as DNA molecular size marker to ascertain the size of the amplified PCR product.

Restriction Enzyme Analysis Of OmpH-PCR Product: The amplified PCR products were subjected to restriction enzyme digestion using restriction enzymes *Dra* I and *Hinf* I. The amplicons from the vaccine and the duck isolates were digested with restriction endonucleases *Dra* I and *Hinf* I.

The digestion was carried out by a standard method.

(Sambrook *et al.*, 1989). Briefly restriction enzyme digestion was carried out at 37°C for two hours, followed by inactivation of the enzyme at 80°C for 20 min. Electrophoresis of the resultant digest was conducted in a 8% cent acrylamide gels, along with undigested PCR product and pBR322/*Alu* I digest molecular size marker. Gels were viewed on a transilluminator and photographed.

Results and Discussion

The primers successfully amplified the *OmpH gene* from template DNA prepared from the boiled culture lysates as well those prepared from the vaccine. The amplification generated a product of about 1000 bp (Fig. 1). Similar results were reported by Luo *et al.* (1999) who had reported successful amplification of *OmpH* genes of the serotypes 1, 3 and 4 to 16 of *P. multocida* and the product had an approximate size of 1 kilo-base pairs.

On comparison the fragments obtained by *Hinf* I digestion of PCR products from DP1 and the vaccine (Cholerin triple) nine bands ranging in size from (900-72 bp) were seen in the restricted amplified product of vaccine, of which three were having the same size as that of duck isolate DP1.

Analysis of the fragments obtained by *Dra* I digestion revealed the presence of eight bands (ranging in size from 900-131 bp) in the restricted amplified product of vaccine, of which four were of the same size as that of duck isolate DP 1 (Fig. 2).

Extraction of template DNA from inactivated oil adjuvant fowl cholera vaccine, using isopropanol was found to be a simple rapid and efficient. The template DNA prepared was found to be adequate for performing PCR. This technique can be used for other bacterial vaccines.

Maas *et al.* (2003) used isopropyl myristate to extract New Castle disease (ND) virus from inactivated ND oil emulsified vaccine. They used the antigens extracted for quantification of haemagglutinin-neuraminidase (NH) and fusion (F) proteins of NDV in oil-adjuvanted vaccines.

Cholerin Triple is a trivalent fowl cholera vaccine incorporating serotypes A:1, A:3 and A:4. The duck isolate DP 1 has been serotyped as A:1 by IVRI, Izatnagar. Similar sized restriction fragments generated by enzymes, viz., *Dra* I and *Hinf* I in both the duck isolate DP1 and the vaccine suggests a high degree of homogeneity of the *OmpH* gene of duck isolate DP 1 as compared to those of the vaccine.

Several studies have indicated that OmpH is an important immunogen of *P. multocida*. Native OmpH was shown to be able to induce protective immunity in chicken against homologous challenge. (Luo *et al.*, 1999)

From the results of the present study it can be concluded that *OmpH* gene of the local duck isolate DP 1 and the A:1 serotype incorporated in the vaccine are similar. Thus the vaccine (Cholerin Triple) can be expected to confer immunity to ducks in Kerala against pasteurellosis.

The additional fragments observed in the restriction profile of *Dra* I and *Hinf* I on the OMPH-PCR product derived from the vaccine is probably attributable to the two other serotypes viz., A:3 and A:4 present in the vaccine. To the best of our knowledge this is the first report of the use of isopropanol for the preparation of template DNA from inactivated oil adjuvant vaccines, for use in PCR.

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