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Detection of *Pasteurella multocida* in experimentally infected embryonated chicken eggs by PCR assay

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Applicability of polymerase chain reaction (PCR) assay to detect *Pasteurella multocida* in experimentally infected embryonated chicken egg was assessed in the present study. PCR assay rapidly and specifically detected the genome of *P. multocida* in amniotic fluid, allantoic fluid and homogenates of infected embryo and its membranes. The sensitivity of detection was as low as 20 bacterial cells/ml of allantoic or amniotic fluids. Detection of *P. multocida* in dead embryos by PCR was possible up to 6 and 30 days or more following storage of dead embryos at 37°C, and at 4°C as well as at -20°C, respectively. The study revealed that PCR assays could be employed directly for detection and confirmation of *P. multocida* infection in experimentally infected chicken embryos.

Keywords: Chick embryo, *Pasteurella multocida*, PCR

The fertile chicken egg has been widely used as a laboratory model for studying the pathogenicity and isolation of microbial agents from suspected clinical specimens. There are reports, which describe use of embryonated chicken eggs for studying the pathogenicity of *Pasteurella multocida*[1-2]. *P. multocida*, a causative agent of fowl cholera, is known to affect all species of domesticated and wild birds. Several strains of *P. multocida* serogroup-A which are known to vary greatly in terms of pathogenicity, are predominantly involved in peracute and acute form of the disease inflicting major economic losses to the poultry industry[3]. Although, experimental mouse and chicken inoculation has been commonly employed method in testing their pathogenicity, this may not be feasible in screening large number of strains. In such cases embryonated chicken eggs appear to be a better option. However, confirmation of embryo mortality following experimental infection usually involves isolation of organisms by culture methods that takes a few days. Moreover, results from several growth media like dextrose starch agar containing 5% chicken serum or other suitable media/blood agar[3] or selective media[4], some times could be disappointing as some media will not support the growth of all strains of *P. multocida*. In that case, rapid detection of *P. multocida* genome by PCR would greatly ascertain the mortality of embryo due to inoculated strain.

There are several studies on detection of *P. multocida* by PCR assays from pure culture, mixed cultures, direct bacterial colony, morbid tissue materials, clinical and environmental samples[5-9]. In the present study, we report detection of *P. multocida* in experimentally infected chick embryo by PCR assay.

Materials and Methods

Bacterial cultures — *Pasteurella multocida* serogroup A:1, *Salmonella gallinarum, Mycoplasma gallisepticum*, and *Escherichia coli* cultures maintained at the Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, were used in the present study.

Experimental infection of chick embryo — Eleven-day-old embryonated chicken eggs collected from a single line of breeder flock, were procured from the Hatchery Unit, Central Avian Research Institute, Izatnagar. *P. multocida* serogroup A:1 was grown in brain heart infusion (BHI) broth (Himedia, India) for 18 hr at 37°C. Ten-fold serial dilutions were prepared in phosphate buffered saline (PBS) and the number of colony forming units (CFU) were determined. Groups of 4 embryonated chicken eggs were inoculated by chorio-allantoic membrane (CAM) route with 0.1 ml inoculum of each dilution containing approximately

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2.5×10^{10} to 25 CFU per egg. A control group of 4 embryos were inoculated with 0.1 ml of sterile BHI broth. Separately, mixed broth culture containing *P. multocida* serogroup A:1, *S. gallinarum*, *M. gallisepticum*, and *E. coli* was also used to infect a group of embryonated chicken eggs by CAM route.

All the eggs were incubated in a humidified chamber and candled twice daily for mortality, if any. All dead embryos together with control embryos were harvested and examined for gross lesions and materials were collected aseptically for further processing. A loopful of sample from each part of the embryo was also inoculated on blood agar plates for confirmation by conventional culture method.

Preparation of template for PCR assay — The allantoic and amniotic fluids were collected from dead embryos by syringe and needle. About 1.5 ml of fluid was taken in a sterile microfuge tube and centrifuged at 10,000 *g* for 10 min. The pellet was resuspended in 500 µl of TE buffer (10m*M* Tris-HCl *p*H 8.0, 1 m*M* EDTA, *p*H 8.0). The lysate was prepared by boiling for 10-15 min followed by immediate chilling for 30 min and 10 µl of the supernatant was used as template for PCR.

Whole embryo and membranes were also collected separately and washed thrice thoroughly with PBS. Approximately 1g of embryonic tissue and membrane samples were subjected to homogenization in a tissue homogenizer containing 5 ml of PBS. The homogenate was allowed to settle for 30 min before the supernatant was removed and centrifuged at 10,000 *g* for 10 min. The pellet was resuspended in 500 µl of TE buffer [10m*M*, Tris-HCl (*p*H 8.0); 1 m*M*, EDTA (*p*H 8.0)]. The homogenized tissue lysate was prepared following boiling for 15-20 min and snap chilling for 30 min. The sample (10 µl) was used as template for PCR.

Primers and PCR assay — Two sets of primers specific for *P. multocida* species and *P. multocida* serogroup-A were used in the present study. The sequences of primers were as follows - KMT1 SP6 (5′-GCT GTA AAC GAA CTC GCC AC-3′) and KMT1 T7 (5′- ATC CGC TAT TTA CCC AGT GG-3′) for species specific (9); RGPMA5 (5′-AAT GTT TGC GAT AGT CCG TTA GA-3′) and RGPMA6 (5′-ATT TGG CGC CAT ATC ACA GTC-3′) for serogroup-A specific 2 amplifications.

PCR amplification was performed in a final volume of 25 µl. The amplification reaction mixture contained 10×PCR buffer [50m*M*, KCl; 10m*M*, Tris-HCl (*p*H 8.3); 1.5m*M*, $MgCl_2$], 200 µ*M* each of dNTPs, 0.5 µ*M* of each primers, 10 µl of template solution and 2 U of *Taq* DNA polymerase (MBI Fermentas, USA). The reaction mixture were placed into a Master cycler (Eppendorf, Germany) and amplified by initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation (95°C for 45 s), annealing (55°C for 45 s), extension (72°C for 45 s), and a final extension step at 72°C for 6 min. Genomic DNA from reference strain was also included with each reaction as positive control. PCR assay for all the samples was performed in duplicates. The amplified products were electrophoresed in1.5 % agarose gel (in 0.5x TBE buffer with 0.5µg/ml of ethidium bromide) and visualized under UV light and photographed (Alpha imager, Germany).

Sensitivity of PCR on infected embryo — A ten-fold serial dilutions of *P. multocida* inoculums were prepared to spike embryonated chicken egg. Each inoculum of *P. multocida* containing 2×10^9 to 2 cells/0.1 ml was used to spike each material (0.9 ml) separately having allantoic fluid, amniotic fluid, homogenates of embryonic tissues and its membranes. All spiked materials were processed as described previously for PCR assay.

To assess the effect of temperature and duration of storage on sensitivity of PCR based detection, a total of 30 chick embryos were inoculated with 0.2 ml of undiluted BHI broth culture of *P. multocida* serogroup A:1 by CAM route. All dead chick embryos were divided into three groups. Each group of dead embryos were stored at 37°, 4° and -20°C, respectively. Chick embryos stored at different temperatures were harvested on 3, 6, 10, 15, 20, 25 and 30 days after death and processed for detection of organisms by cultural method as well as by PCR.

Results

Detection of P. multocida in experimentally infected chick embryo by PCR — PCR detected *P. multocida* serogroup-A in allantoic fluid, amniotic fluid and homogenates of embryo and its extra embryonic membranes by producing good intensity amplicons of expected size. Amplified products of ~460 bp and ~564 bp were observed in PCR using species specific and serogroup-A specific primers, respectively (Fig.1). PCR gave negative amplification in those infected embryo materials which were mixed with yolk. However, a positive amplification was observed after thorough washing of embryo and its

membranes by PBS prior to homogenization. Similar PCR results were also observed in infected chick embryos with mixed culture.

Sensitivity of PCR on chick embryo samples — PCR assay found to detect as low as 20 cells/ml in both the allantoic and amniotic fluids. Whereas, the limit of detection in homogenized embryo and extra embryonic membranes were found to be approximately 2×10^2 cells. Detection of *P. multocida* by PCR in dead chick embryos gave positive amplification from all the samples tested up to 6 days following storage at 37°C. Since, dead embryos were putrefied by 7 days onward, they were discontinued from the study. PCR also detected *P. multocida* in the embryos stored at 4°C as well as at -20°C even after 30 days of storage (Table 1). Amplification was possible using both the primers for all the samples of embryo tested at different intervals.

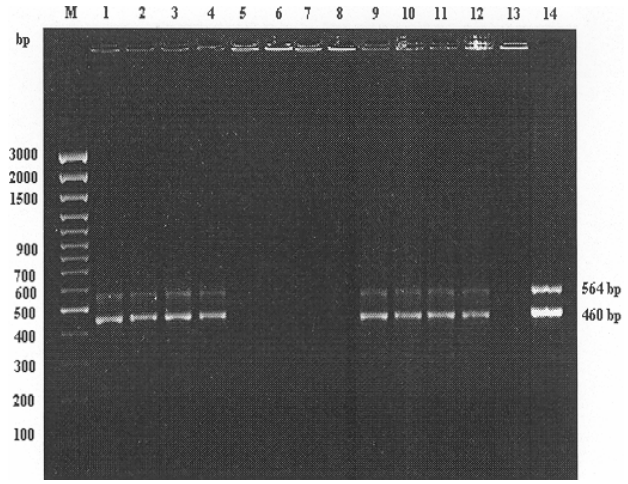

Fig.1 — Multiplex PCR on infected chick embryo [Lane M- 100 bp DNA ladder plus marker; Lane 1-Allantoic fluid; Lane 2-Homogenate of embryo membranes; Lane 3-Amniotic fluid; Lane 4- Homogenate of embryo body; Lane 5 to 8- Samples order same as above but mixed with yolk; Lane 9 to 12- Samples order same as above but from Mixed culture infected chick embryo; Lane 13-Uninfected chick embryo sample as template; and Lane 14-Genomic DNA of *P. multocida* as template]

Discussion

Our findings indicated that PCR can be successfully applied for detection of *P. multocida* serogroup-A in experimentally infected chick embryo using species specific and serogroup-A specific primers. There was no failure in amplification from embryo materials, indicating the absence of PCR-inhibitory factors in allantoic, amniotic fluids, embryo and its membranes. But, embryonic materials mixed with yolk, hampered PCR amplification. Its inhibitory effect was also confirmed by absence of amplified product from fluids mixed with yolk. However, this limitation could be successfully eliminated by washing embryonic material thoroughly with PBS prior to homogenization.

There was no non-specific amplification by PCR when applied on chick embryos infected with mixed cultures. PCR performed with allantoic and amniotic fluid gave the best results with high sensitivity and reproducibility. In addition, species specific and serogroup-A specific PCR assays do not cross react with other bacteria[6,9]. The present study indicated that the sensitivity of PCR assay was as low as 20 CFU in amniotic and allantoic fluid. Moreover, there was no loss of bacterium during the processing as the fluids were directly used for template preparation. However, lower detection limit of PCR in homogenized embryo and membrane could be due to loss of bacterium during homogenization and further processing.

This appears to be the first report on application of PCR for detecting *P. multocida* from experimentally infected chick embryo. The described method was rapid, less labour intensive and more sensitive over the traditional culture method. Furthermore,

Table 1-Amplification of specific amplicon of *P. multocida* serogroup A from morbid materials kept at different temperature and time interval

Name of Specimen	Days/Temperature (^{0}C)															
	3			6			10		15		20		25		30	
	37	4	-20	37	4	-20	4	-20	4	-20	4	-20	4	-20	4	-20
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A- Allantoic fluid; B- Amniotic fluid; and C- Homogenized embryonic tissue and membranes

(+): Denotes amplification of amplicon of *P. multocida* by species specific and serogroup A specific PCR assay

(-): Denotes no amplification of amplicon

The PCR assay using chick embryos were discontinued after the 6th day on account of the samples being putrefied, which is already mentioned in the result part of the text

purification of DNA by phenol extraction or pre-enrichment in broth culture was not necessary to yield reproducible results from infected chick embryo. Confirmatory test can be repeated several times on the same extra-embryonic fluids even after storage for a long time. As fowl cholera is predominantly caused by strains of *P. multocida* serogroup-A, simultaneous detection of species and serogroup-A in a Multiplex PCR without using a separate reaction mixture will be desirable especially in screening the pathogenecity of large number of *P. multocida* serogroup-A strains in chick embryos.

The current study revealed that direct application of PCR assay on infected embryonic materials could drastically reduce the time required for confirmation of *P. multocida* strains infectivity to embryonated eggs. In light of the present observation the clinical materials from fowl cholera cases can directly be used for diagnosis employing PM-PCR and serogroup-A specific primers. Diagnosis based on PCR assay is easy to perform and less time consuming over conventional method.

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