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Short communication

PCR assay for rapid detection of *Pasteurella multocida* serogroup A in morbid tissue materials from chickens with fowl cholera

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Fowl cholera is a commonly occurring avian disease that affects all species of domesticated and wild birds. Several strains of *Pasteurella multocida* serogroup A are predominantly involved in the peracute and acute forms of the disease, inflicting major economic losses to the poultry industry (Rimler and Glisson, 1997). A presumptive diagnosis of fowl cholera may be based on the occurrence of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues such as blood, liver or spleen. However, the confirmation mainly depends on isolation and identification of the causative bacterium *P. multocida*, from infected birds. Serological tests such as agar gel immunodiffusion (AGID), indirect haemagglutination test (IHA), and enzyme-linked immunosorbent assay (ELISA) are rarely used for diagnosis.

The complexities associated with the diagnosis of fowl cholera by conventional methods of isolation, identification, capsular serotyping and differential diagnosis from other bacterial diseases such as salmonellosis, collibacillosis and listeriosis in chickens have been largely superceded by DNA-based methods, which are sensitive and specific for rapid diagnosis and help to initiate early preventive measures.

Recently, a new primer set, designed by Gautam (2002) to develop a polymerase chain reaction (PCR) assay for detecting *P. multocida* serogroup A isolates, was identified within genes *hya*C–*hya*D of region 2 capsule biosynthetic locus of *P. multocida* A:1. This PCR assay was known to produce an amplified product of ~564 base pairs (bp) with high specificity and sensitivity, and can be employed for rapid detection from bacterial colonies and bacterial culture lysate.

In the present report we describe the use of a *P. multocida* serogroup A specific PCR assay for the diagnosis of fowl cholera by evaluating its direct applicability on tissue samples of spleen, lung, liver, heart, and bone marrow, collected from experimentally infected chickens. We also attempt to describe a simple and rapid method of detection without involving extensive procedures for DNA extraction or enriched culture lysate preparation methods. The method's diagnostic efficacy was compared with that of in vitro culture.

A total of 16 healthy White Leghorn chickens, 12 weeks of age and negative for antibodies against *P. multocida* as tested by agar gel precipitation test (AGPT), were used. The birds were divided into four groups with four in each group. The birds were inoculated with 1.5 mL of 18 h brain heart infusion (BHI) broth culture of *P. multocida* serogroup-A:1 containing approximately 1.35×10^9 colony forming units (cfu)/mL, by intranasal, subcutaneous or intramuscular routes and one group served as control. The birds were maintained in separate isolation units and given feed and water ad libidum were observed up to eight days. The study was approved by the Committee for the Purpose of Control and Supervision of Experiments on

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From each dead bird, samples of lung, liver, heart, spleen, and bone marrow from long bones (e.g. femur and tibia) were collected for further processing. In addition, tissue samples were collected from sacrificed healthy birds, and included as negative controls in tissue processing and PCR detection. Three sets of tissue samples, with each representative sample of approximately 0.2–0.3 g, were excised from two different sites of each organ and processed as follows.

The first set of tissue samples was used for the reisolation of *P. multocida* on blood agar using standard bacteriological procedures and identification by conventional methods as well as by a *P. multocida* specific (PM)-PCR assay conducted on the direct colony (Townsend et al., 1998). A second set of tissues samples was inoculated in to 2 mL of BHI broth and incubated at 37 °C. After 18 h incubation, 1.5 mL of broth culture were taken in a sterile microfuge tube and centrifuged at 10,000*g* for 10 min; the pellet was resuspended and washed twice with 1 mL of phosphate-buffered saline (PBS) and finally dispensed in 200 μL of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0). The culture lysate was prepared by boiling for 10–15 min followed by immediate chilling for 30 min and the supernatant was used as template for PCR.

The third set of tissue samples was homogenized with approximately 1.2 mL 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 homogenized tissue lysate was prepared as described for the culture lysate, but with no extensive DNA extraction procedures. The supernatant was subjected to rapid detection by PCR.

To assess the effect of temperature and duration of storage on sensitivity of PCR-based detection, different tissue samples from each dead bird were divided into three groups. Each group of tissue samples was stored at 37, 4 and −20 °C, respectively. Tissue samples were processed immediately for detection of organisms by culture as well as by PCR, after collecting morbid materials (zero day) and subsequently on 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 40, 50, 60, 80 and 100 days post-storage at different temperatures. The statistical analysis was carried out following standard methods of Snedecor and Cochran (1967).

The oligonucleotide sequences of primers used in the study were forward primer RGPMA5 (5′-AAT GTT TGC GAT AGT CCG TTA GA-3′) and reverse primer RGPMA6 (5′-ATT TGG CGC CAT ATC ACA GTC-3′), binding at the genomic position 6912–6934 and 7475–7455, respectively (Gautam, 2002). PCR for each sample was carried out in a thermal cycler (Eppendorf) in a 25 μL reaction volume. The reaction mixture contained 8 μL of template DNA, 0.5 μL of 10 mM dNTPs mix, 1.5 μL of 2.5 mM $MgCl_2$ with 2.5 mL of 10× PCR buffer (MBI Fermentas), 20 pmol of each primer (Bangalore Genei) and 2 U of *Taq* DNA polymerase (MBI Fermentas). Standard amplification conditions included initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation (95 °C for 45 s), annealing (56 °C for 45 s), extension (72 °C for 45 s), and a final extension step at 72 °C for 6 min. PCR assay for all the samples was performed in duplicates. Control genomic DNA from the reference strain was also included with each reaction. The amplified product was visualized by electrophoresis using 10 μL of the final reaction mixture on a 1.5% agarose gel.

The results of PCR assay using different template DNA preparations gave amplified bands of identical size (Fig. 1). PCR assay on direct homogenized tissue lysate indicated an amplified product of ~564 bp from tissue samples of all the organs tested. These results correlated well with the results of PCR assay conducted on culture lysates prepared following tissue enrichment and conventional methods confirming the reisolation of *P. multocida* from the tissues. Our results were consistent each time they were tested indicating the reproducibility of the results using this technique. All culture positive specimens were also PCR positive. Compared with *P. multocida* cultivation, PCR assay was less laborious and results were available within 6 h. The amplicon produced by directly homogenized tissue lysate prepared from bone marrow was intense in comparison to rest of the tissues. However, in comparison to the bacterial culture lysate, the PCR band amplified directly from homogenized tissue lysate was often faint. This could be due to loss of bacterium during the processing or due to small number of *P. multocida* cells in tissue samples and a smaller quantity of genomic DNA in the supernatant of homogenized tissue lysate.

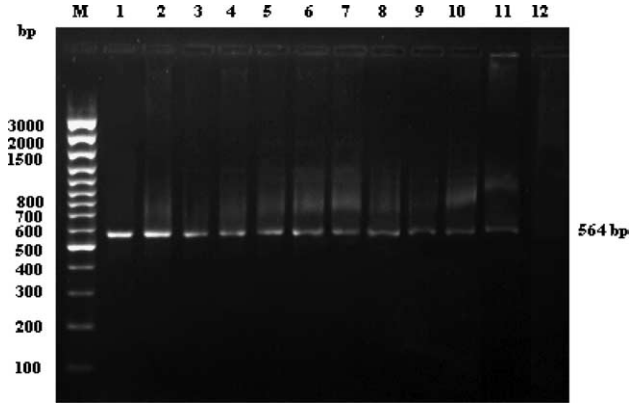

Fig. 1. *Pasteurella multocida* serogroup A specific PCR. Lane M: 100 bp DNA ladder plus; lane 1: positive control (genomic DNA); lane 2: bone marrow; lane 3: liver; lane 4: spleen; lane 5: lung; lane 6: heart (enriched tissue culture lysates); lane 7: bone marrow; lane 8: liver; lane 9: spleen; lane 10: lung: lane 11: heart (homogenized tissue lysates); lane 12: negative control.

The detection limits of the PCR assay of *P. multocida* in different materials stored at various temperatures are given in Table 1. Morbid materials gave positive amplification from all samples tested when tissues were processed immediately and PCR assay was employed within 24 h after collection of materials. Following storage at 37 °C, detection was possible only up to three days post-storage. On further storage, the detection was hampered by putrefaction. PCR also detected *P. multocida* in tissues stored at 4 °C as well as at −20 °C even up to 40 days of storage. However, the reproducibility of results from tissues varied greatly due to the lack of reproducibility except for bone marrow, which showed good reproducibility. The sensitivity of PCR on stored morbid materials is presented Table 2. The χ^2 value (at 1% significance) showed that PCR was sensitive on morbid materials stored at different temperatures. The application of PCR in bone marrow was particularly satisfactory giving an amplified product even after 100 days post-storage at −20 °C. However, the reproducibility in other tissues was not satisfactory possibly due to inhibitory/interfering substances, the type of tissue selected for processing, the method of processing and storage etc.

PCR assay for either purified *P. multocida* DNA or enriched tissue materials with BHI broth before preparation of culture lysates have recently been reported (Lee et al., 2000). The method described here for the identification of *P. multocida* serogroup A directly from homogenized tissue lysates prepared from different organs without enrichment of tissues in BHI broth or involvement of DNA extraction from tissues by cell lysis followed by phenol:chloroform:isoamylalcohol extraction method, is very simple and rapid in comparison to conventional methods of detection and capsular serotyping. The technique is much faster than bacteriological culture and reduces the time for diagnosis from a few days to 6 h. Moreover, the rapid detection of *P. multocida* in morbid materials assumes a practical importance due to the fact that in most fowl cholera outbreaks (peracute and acute), birds are found dead.

Table 1
Detection of *P. multocida* serogroup-A in morbid materials of chicken following storage at different temperatures by PCR assay

Sl. no.	Storage temperature	Organ	Post-storage intervals (days)															
			0	1	2	3	4	5	7	10	15	20	25	30	40	60	80	100
1	37 °C	BM	12/12	11/12	9/10	2/8	–	–	–	–	–	–	–	–	–	–	–	–
		Liver	12/12	9/12	6/12	0/12	–	–	–	–	–	–	–	–	–	–	–	–
		Spleen	12/12	10/12	7/12	1/10	–	–	–	–	–	–	–	–	–	–	–	–
		Heart	12/12	9/12	4/12	1/12	–	–	–	–	–	–	–	–	–	–	–	–
		Lung	12/12	10/12	3/12	0/12	–	–	–	–	–	–	–	–	–	–	–	–
2	4 °C	BM	12/12	12/12	12/12	11/11	11/11	10/10	9/9	9/9	8/8	7/7	6/6	6/6	5/5	3/3	3/3	2/2
		Liver	12/12	10/12	9/12	9/12	8/12	6/12	5/12	5/12	6/12	4/12	4/12	3/12	2/12	0/12	0/12	0/12
		Spleen	12/12	10/12	10/12	11/11	10/11	9/10	9/10	8/9	6/9	6/8	4/6	2/4	1/3	0/2	–	–
		Heart	12/12	9/12	9/12	8/12	9/12	8/12	7/12	6/12	4/12	4/12	3/12	1/12	0/12	0/12	0/10	0/10
		Lung	12/12	9/12	8/12	8/12	7/12	6/12	6/12	5/12	5/12	4/12	3/12	3/12	0/12	0/12	0/12	0/10
3	−20 °C	BM	12/12	12/12	12/12	12/12	10/10	9/9	9/9	8/8	8/8	8/8	7/7	6/6	4/4	4/4	2/2	2/2
		Liver	12/12	10/12	10/12	9/12	10/12	9/12	8/12	8/12	7/12	6/12	6/12	3/12	0/12	0/12	0/12	0/12
		Spleen	12/12	11/12	11/12	10/11	9/11	8/10	8/10	7/9	6/9	4/7	3/5	2/4	1/3	0/3	0/3	0/2
		Heart	12/12	9/12	8/12	9/12	9/12	9/12	9/12	7/12	7/12	5/12	4/12	2/12	1/12	0/12	0/10	0/10
		Lung	12/12	10/12	8/12	9/12	9/12	8/12	8/12	8/12	7/12	6/12	6/12	7/12	2/12	0/12	0/12	0/12

•, Number of positive amplifications/number of samples analysed; –, No samples were analysed.

Table 2
Sensitivity of PCR on stored morbid materials

Sl. no.	Storage temperature		Organs					χ^2 value
			BM	Liver	Spleen	Lung	Heart	
1	37 °C	+/NS	37/42	27/48	30/46	25/48	26/48	17.10*
		%	80.5	56.25	65.21	52.08	54.17	
2	4 °C	+/NS	125/125	83/192	98/119	76/190	80/188	14.06*
		%	100	43.22	82.35	40.0	42.55	
3	−20 °C	+/NS	126/126	98/192	92/123	100/192	91/188	40.79*
		%	100	51.04	74.79	52.08	48.40	

+/NS denotes positive amplification/number of samples analysed. *Significant at 1% level.

Our method involving a *P. multocida* serogroup A PCR assay is simple, rapid and reproducible and is a promising diagnostic tool for confirming the presence of *P. multocida* serogroup A directly from morbid materials especially from bone marrow of long bones without in vitro cultivation.

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