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PCR DETECTION AND IDENTIFICATION OF AVIAN *PASTEURELLA MULTOCIDA* IN CLINICAL SAMPLES BASED ON THE KMT_1 SEQUENCE

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SUMMARY

Polymerase Chain Reaction (PCR) was used for the detection and identification of *Pasteurella multocida* (PM) in poultry clinical samples with the KMT_1SP_6/ KMT_1T_7 primer pair. A total of 54 clinical samples (liver, spleen, cloacae swabs) were assayed and finger prints of 14 samples corresponded with the 460bp expected band size. Results were unambiguous as finger prints were single and distinct. The sensitivity test of the KMT_1SP_6/ KMT_1T_7 primer pair gave 10^{-5} dilution (0.03025µg) as the limit of detection using ethidium bromide staining of amplified DNA in agarose gel electrophoresis. The PM - PCR was specific and reproducible. Southern hybridization results confirmed the PM - PCR results and have therefore been shown to be a sensitive and reproducible confirmatory assay. The KMT_1 sequence based primers (KMT_1SP_6/ KMT_1T_7) proved sensitive and reliable to be used for the detection and identification of PM in clinical samples.

KEY WORDS: *Pasteurella multocida*; PCR; southern hybridization; fowl cholera.

INTRODUCTION

Pasteurella multocida (PM), stereotype A is the causative agent of fowl cholera in poultry and of the 16 somatic types, A_1 A_3 and A_4 have been shown to be the most prevalent (Gunawardana *et al.*, 2000).This disease is still reported to be endemic in regions of Asia and Africa where it is of great economic importance due to loss of poultry birds (Townsend et al., *1997*; Muhairwa *et al.*, 2000). In Nigeria a great loss in poultry production as a result of this disease is still recorded.

Due to the peracute nature of this disease, definitive and accurate diagnosis is critical to effective control, as it allows for rational deployment of limited resources such as vaccine and animal health expertise. In Nigeria, definitive diagnosis is still dependent on isolation and identification of the causative agents. Under field conditions, the opportunity for good quality bacteriological submissions reaching the laboratory for processing is still a limiting factor. Delays in collection or processing bacteriological samples may result in death of the organism or overgrowth by contaminants. These conventional methods involve exhaustive task of obtaining pure cultures which is also time consuming.

The development of various nucleic acid based assays has dramatically improved the sensitivity, specificity and versatility of bacterial detection and identification (Townsend *et al.*, 2000; Townsend *et al., 1997*). Genetic techniques such as PCR are receiving increased application in veterinary medicine for the diagnosis of infectious diseases such as fowl cholera. PCR now plays a critical role in the clinical laboratory as rapid and specific detection of micro-organisms have provided remarkable advances in the diagnosis of infectious agents, particularly in cases where the presence of an organism has significance (Relman and Persing, 1996).

The application of PCR to directly detect PM from clinical samples has allowed PCR to be used as a diagnostic tool for fowl cholera (Nih, 1992; Townsend *et al.*, 2000). Hunt *et al* (2000) in a review, rated the sensitivity of *P. multocida* specific PCR assay high (detecting < 10 organisms) particularly, the PM PCR based on the amplification of the KMT_1 sequence. The development of PM-PCR will provide presumptive identification of all PM species and hence can be used to detect PM directly from clinical samples, allowing for speedy, accurate or definitive identification of the causative agent. These in turn translates into speedy diagnostic conclusion and hence prompt control measures instituted. PM-PCR is also a valuable tool for rapid epidemiological analysis and determination of out break-related cases.

PM-PCR assay has been centered on ps1 gene encoding the P6 - like protein of PM and the KMT_1 sequence which is unique to PM (Hunt *et al*, 2000). The KMT_1 sequence obtained by subtractive hybridization has been shown to serve as a basis for PM group specific detection and its amplification unambiguous (Townsend *et al,* 1998a). In this study PM-PCR using the KMT_1SP_6/ KMT_1T_7 primer pair was used to detect and identify PM in clinical samples collected in Veterinary clinics and poultry farms in Jos and environs. A speedy, definitive, and accurate detection and identification technique for PM was developed. Southern hybridization was used as a confirmatory technique to the PM-PCR.

MATERIALS AND METHODS

Samples

Three serotypes of PM (type A from chicken, types B and E from cattle, type A from quail) grown in brain heart infusion (BHI) broth culture were collected from Bacterial Research Department, National Veterinary Research Institute (NVRI), Vom. A total of 54 samples, comprised of thirty six poultry tissue samples (liver and spleen) collected from veterinary clinics in Jos and environs, NVRI poultry farm and the Diagnostics Department of NVRI Vom; and eighteen cloacae swabs were collected from two farms in Vom. Samples were stored at -20°C until homogenized for DNA extraction

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Genomic DNA extraction

Genomic DNA was prepared essentially as described by Silhavy *et al* (1984). One and a

half milliliters of broth culture was centrifuged at 10, 000 RPM for 5minutes and the pellet washed once with sterile distilled water and reconstituted with 500µl T. E. Buffer (pH8.0). Five hundred milligrams (500mg) of each tissue sample was weighed into a mortar and homogenized with the aid of sterile sand and reconstituted with T. E. buffer (pH8.0). Digestion was with 50µl (50mg/ml) lysozyme (sigma) and 100µl (1mg/ml) of proteinase K (Promega Madison, WI, USA) for 4-18 hours. DNA was extracted three times with phenol: chloroform: isoamyl alcohol (25:24:1). The DNA was precipitated with absolute ethanol (BDH), dried and re suspended in 30 50µl nuclease free water (Promega, Madison, WI, USA). Bacterial cells were washed twice and re-suspended in 50ul nuclease free water and used as template for PM-PCR.

***Pasturella multocida* specific PCR (PM-PCR)**

PM - PCR assay was performed using the KMT_1 sequence based primers as designed by Townsend *et al* (1998a). The forward primer (KMT_1SP_6), 5'-GCTGTAAACGAACTCGCCAC-3' and the reverse primer (KMT_1T_7), 5'-ATCCGCTATTTACCCAGTGG-3' were synthesized by Roche, Mannheim, Germany. A 5.0µl genomic DNA extract or bacterial suspension was used as template with Mg^{2+} free 2.5µl 10×PCR reaction buffer (Promega, USA), 0.5µl 20mM dNTPs, 2.0µl of 25mM $MgCl_2$, 1.0µl of each primer (10pmole); 2.5 units of Taq DNA polymerase (Promega, USA) in a total volume of 25µl with 30-50µl mineral oil overlay. PM - PCR amplifications were performed in an automated Cyclogene thermo cycler (Techne®, Cambridge, U.K) with initial denaturation at 95°C for 4 minutes. This was followed by 30 cycles of denaturation at 95°C for one minute; annealing at 55°C for one minute; and extension at 72°C for one minute. Final extension was at 72°C for nine minutes. Ten micro liters of PCR product was electrophoresed in an agarose gel (1.5%) containing 10µl of 10mg/ml ethidium bromide at 60 80 volts for 45 60 minutes. One hundred base pair marker (Roche, Mannheim, Germany) was used as a molecular size marker. DNA amplifications were examined by U.V trans- illumination and photographed using Polaroid MP-4 land camera.

Southern hybridization

Bands corresponding to the expected amplicon size (460 bp) from PM- PCR amplifications of the serotype A reference material were excised from agarose gels and purified using Agarose gel DNA Extraction kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly: agarose was solubilized using 300µl of the agarose solubilization buffer per 100 mg of agarose. 10µl of silica suspension was added to capture DNA. The matrix was suspended in 500µl nucleic binding buffer and centrifuged to obtain pellet containing DNA. Nuclease free water (Promega, USA) was used to elute DNA by vortexing and incubation for 10 minutes at 56°C.

The purified amplicons were labeled using the DIG Labeling and Detection starter Kit I (Roche, Germany) and the random prime labeling protocol according to the manufacturer's instructions. Fifty micro liters (50µl) of the DNA template was denatured at 95°C in a heating block for 10 minutes. The DIG high Prime mix (Digoxigenin-11- d UTP, hexamers, Klenow polymerase enzyme and an optimized reaction buffer) was added and incubated over night at 42°C. DNA bands on agarose gels were blotted on to nylon membranes by down ward capillary transfers overnight according to the modified method of Koetsier *et al* (1993). Fixation was by baking in a micro wave oven (National) at full power for 3 minutes. Pre hybridization of the membrane was carried out for 30minutes at 42°C in 20ml of hybridization buffer, DIG-Easy Hyb (Roche Germany) with gentle agitation. Labeled probe DNA was denatured at 95°C for 5minutes and added to 30ml hybridization buffer (DIG - Easy Hyb) and mixed well. Probe/hybridization buffer mixture was added to the membrane in a plastic container with lid and incubated overnight at 42°C with gentle agitation in a hybridization incubator (GFL, Germany). Stringency washes were carried out with

2×SSC, 0.1% SDS, 0.5×SSC, 0.1% SDS twice at room temperature and at 65°C respectively with constant agitation. Detection was with Anti-Digoxigenin AP and NBT/BC IP colour substrate (Roche). Results were visualized as brown blue color development and photographed with black and white film.

PCR sensitivity and specificity tests

PCR sensitivity test was determined by growing a broth culture of serotype A in BHI overnight at 37°C. From 1.5ml aliquot of the culture DNA was extracted using Tri pure Reagent (Roche). Sensitivity of the test was determined using 10-fold serial dilutions of the extracted DNA. Starting with 10µl, the quantity of genomic DNA used for amplification was estimated spectrophotometrically at A260 using Amersham Pharmacia ultrospec 4000, UV/Visible spectophotometer.

The number of organisms represented by a quantity of genomic DNA was estimated using the molecular mass of E. coli as the unit measure (3.1×10^{9}). PCR specificity was determined using *Pasteurella haemolytica*, *Brucella abortus* and *Brucella* vaccine strain S19 genomic DNA extract.

RESULTS

A total of 4 reference isolates (serotype A Chicken, serotype A-quail, serotypes B and E cattle), and 54 clinical samples were assayed. It was determined that comparisons of PM - PCR finger prints should be based on amplified fragment of 460bp in length detected after 30 cycles as carried out by Townsend *et al* (1998a). The Silhavy *et al* (1984) and the Boom *et al* (1990) genomic DNA extraction methods among the methods tried were found to be more reliable, reproducible and gave high DNA yields. The PM - PCR assay of the four reference samples was carried out with the KMT_1SP_6/ KMT_1T_7 Primer pair and single bands of expected size after agarose gel electrophoresis and ethidium bromide staining of the PCR product were obtained (fig. 1). Comparison of the finger-prints showed that bands obtained were at the same position (460 bp) when measured against 50bp marker (Roche, Germany). The three serotypes available (A, B. and E) though not quite a wide range of serotypes demonstrated a species - specific band of approximately 460bp.

Direct use of washed PM cells as templates for amplification equally gave distinct bands as the extracted DNA. Five microliters aliquots used as templates gave clear and distinct bands. Optimization result shows that amplification was best with 0.5µl (10pmole) primers, 0.5µl (5units/µl) Taq polymerase and 5µl DNA extract. Good bands were obtained with resultant reduction in the appearance of artifacts. One observation that is strikingly apparent is the appearance of the expected bands for the different serotypes (A, B, E) at the same position (460 bp) indicative of a common KMT_1 sequence in the PM species as established by Townsend *et al* (1998a).

The 54 clinical samples were from fourteen flocks two of which were quail flocks. Eighteen of the samples were cloacae swabs from 2 flocks which based on post mortem examination were diagnosed to have fowl cholera. However, phenotypic characterization showed that the organism was suggestive of *Pasteurella haemolytica*. Isolates from organs of clinically sick and dead chickens from these flocks showed same serological and biochemical characteristics as earlier reported by Antiabong *et al* (2005). PM-PCR of the isolates was carried out using KMT_1SP_6/ KMT_1T_7 primer pair and there was no amplification. Fourteen of the 54 samples were positive for the group specific PCR (PM-PCR). Figure 2 depicts the PM-PCR of six clinical samples (tissues) analyzed following the same pattern as the PM reference materials. There were no differences observed in the DNA banding Patterns of the clinical samples as all the positive samples gave single and distinct bands at the expected 460bp position. It was found during this study that Taq DNA polymerase and Tth DNA polymerase could be used and the distinctiveness and clarity of the bands was the same. The cycling conditions are as reported by Townsend *et al* (1998a) gave the best bands and hence was maintained through

out this study.

The 100µl of tissue homogenate used for DNA extraction in this study was adequate to give visualized and clear bands under U. V. light except in few cases where bands were not seen but were positive with southern hybridization. This may be as a result of low PM cells in the tissue. Five microliters of the tissue DNA extract gave adequate templates for amplification that was visualized under U. V. light. Three to five microliters of the crude tissue DNA extracts tried in this study gave satisfactory results, except for smears in some rare cases. Re amplification was required for volumes below 3µl and gross smears were observed for volumes above 5µl. 0.3 - 0.5µl (10pmoles) of primers and 0.5µl (2.5 units) of Taq or Tth DNA polymerase gave good bands under existing conditions in our laboratory.

The PM-PCR was used to obtain templates for probe design for southern hybridization analysis. Amplicons of the serotype A reference material was purified for probe making. Southern hybridization was used as a confirmatory technique for the PM-PCR. The digoxigenin (DIG) labeled PCR product was used to probe a southern blot of the positive PM-PCR amplicons together with controls. The southern blots revealed the presence of the amplicons of the positive samples and probe did not hybridize to the negative control (*P. haemolytica*) (Fig.3). These results confirm that the regions amplified in the clinical samples were the same with that of PM reference materials used.

The sensitivity of the KMT_1SP_6/ KMT_1T_7 Primer pair was tested for their ability to detect genomic DNA extracted from 10-fold serial dilutions of washed cells from 1.5ml of serotype A broth culture. The limit of detection using ethidium bromide staining of amplified DNA in agarose gel electrophoresis was the 10^{-5} dilution (0.03025µg) The quantity of genomic DNA (0.03025µg) used for amplification was estimated spectrophotometrically at A_{260}. The number of organisms represented by 0.03025µg of DNA was ~$1.78x10^6$cells and was estimated using the MW of *E. coli* as the unit measure (3.1×10^9cells). Specificity of the PM-PCR was tested using genomic DNA extract of *P. haemolytica*, *B. abortus* and the vaccine strain S_{19} to run PCR along side those of the reference materials (serotypes A, B, E). There were no amplicons detected for *P. haemolytica* or the *Brucella* strains. The specificity of the PM - PCR needs to be evaluated further using genomic DNA templates from other Pasteurella species like *P. langua* and *P. volantium* among others.

DISCUSSION

PCR technique now plays a critical role in the clinical laboratory as a rapid and specific technique for the detection of micro organisms and has provided remarkable advances in the diagnosis of infections agents. PM-PCR assay developed by Townsend *et al* (1998a) is based on the amplification of a DNA sequence unique to *P. multocida* (KMT_1). PM-PCR as applied in this study to detect and identify PM in clinical samples has been shown to be sensitive and reproducible. Comparison of the finger prints of the reference materials and the positive clinical samples did not show any significant difference in the banding patterns especially in their positions as measured against a 50bp or 100bp marker (Roche). Intensities of the bands of the positive clinical samples were comparable to those of the reference materials (Figs1&2). Although comparison of PM-PCR finger prints using a wide range of isolates and serotypes was not performed the results obtained using the four isolates of the 3 serotypes (A, B, E) demonstrated the suitability of the test in PM species detection and identification. There was no observable difference in the bands obtained for the four isolates of three serotypes. The PCR assay confirmed earlier serological and biochemical tests by the Bacterial Research Department of NVRI, Vom especially the isolates from quail which was the first reported case of fowl cholera in quails in Nigeria (Odugbo et al., 2004). This is in line with the report by Hunt *et al* (2000).

As demonstrated in this study, nature of sample was not a hindrance to achieving detectable

amplification as different types of samples (tissue, cell culture, and swabs) gave similar results. Modifications to sample preparation and DNA extraction made the PCR analysis to be performed with ease. For the purpose of detection and identification of PM in clinical samples from different species or breeds often infected by PM (Poultry), PM-PCR has been shown to be reliable, and sensitive. Hence it is a reliable and useful technique in the diagnosis of fowl cholera.

The observed homogeneity in banding patterns among the different serotypes studied, demonstrated that there is no significant difference within species in the KMT_1 and hence can be used to detect presence of PM in any of the samples tested as the KMT_1SP_6/ KMT_1T_7 primer pair has not exhibited disease or serotype specificity. Direct analysis of washed PM cells by PCR was demonstrated. The results demonstrate that cloacae swabs can be sent to another laboratory or received from field out breaks for PM-PCR analysis. Appearance of good amplification obtained when clean PM cells were used as source of template is an added advantage in the speed of PM-PCR analysis. This is because time and reagents used in DNA extraction are saved. This result confirms similar results earlier published by Gunawardana *et al* (2000). While PM PCR may not be able to distinguish between carrier status and clinical infection, clinical samples can be analyzed using PM-PCR to establish fowl cholera. PM-PCR can also be used to investigate carrier state of PM in healthy commercial poultry flocks and flocks affected by fowl cholera. It is also a valuable epidemiological technique as the source of new infection in the flocks may be established.

PM-PCR in this study has been effectively utilized to confirm an earlier postmortem examination and clinical investigation results. Clinical examination and postmortem results pointed to fowl cholera, but *P. haemolytica* was isolated and characterized. PM-PCR using the KMT_1SP_6/ KMT_1T_7 primer pair was not able to amplify, indicative of the absence of PM in the clinical samples and hence confirming the serological and biochemical results that the organism in question is *P. haemolytica* as reported by Antiabong *et al* (2005). In view of the importance of this finding, there is the need to use *P. haemolytica* specific primer pair to confirm this as there is little information available on the pathogenic role of this organism in the avian species. Southern hybridization analysis employed as a confirmatory technique to the PM-PCR has proved reliable, reproducible and effective. Although it may not be cost effective, southern hybridization an older DNA based analytical technique further enhanced reliability of PM detection and hence diagnosis of fowl cholera.

In conclusion, PM PCR in this study has been demonstrated to be a very sensitive, specific and reproducible technique that can be used in the definitive diagnosis of fowl cholera in clinical samples. Direct use of washed cells as templates has boosted the speed with which detection and identification of PM in cultures can be performed. PM-PCR can also be used to investigate carrier state of PM in healthy commercial poultry flocks and flocks affected by fowl cholera. The high sensitivity of the technique demonstrates that cloacae swabs can be sent to another laboratory or received from field out breaks for PCR analysis. It was also evident that the KMT_1 sequence based primers (KMT_1SP_6/ KMT_1T_7) are sensitive and reliable for the detection and characterization of PM in clinical samples.

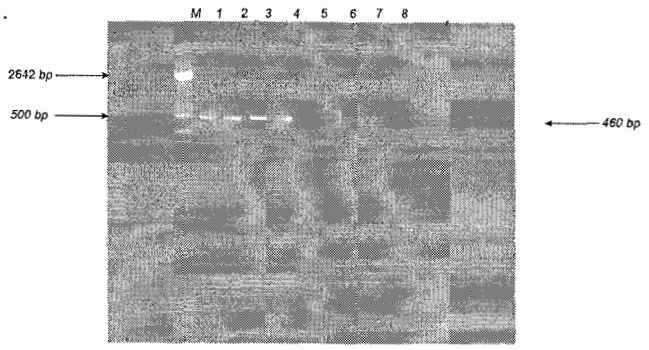

Figure 1:*P. multocida*-specific PCR assay. This figure illustrates finger prints of the 4 reference isolates, amplified by PCR using the KMTSP$_6$ KMT, T$_7$ Primers. M, 50 bp marker (Roche), Lane 1, Serotype A (Chicken), Lane, Serotype A (Quail), Lane 3, (Serotype B (Standard), lane 4, Serotype E (Obudu), Lane 5, Serotype B (repeat), Lane G, +ve control, Lane 7, -ve control, Lane 8, H_20 control

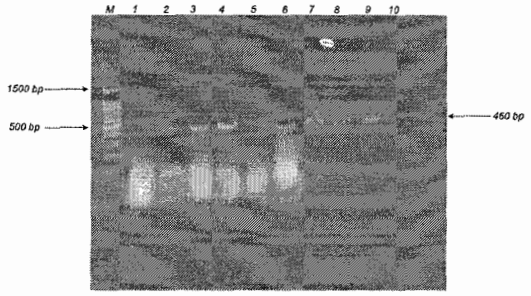

Figure 2: *P. multocida*-specific PCR assay of six clinical samples. This figure illustrates fingerprints of PM PCR of clinical samples (Spleen 2 (ECWA Vet.), Lane 3, spleen (Vet. Clinic), Lane 4, spleen (Vet. Clinic) Spleen 4 (Diagnostics Dept.), Lane 5, Liver, (Diagnostics Dept.), Lane 6, Serotype A (Quail 1), Lane 7, (Quail 2), Lane 8, (Quail 3), Lane 9, +ve control, Lane 10, (H_2O control)

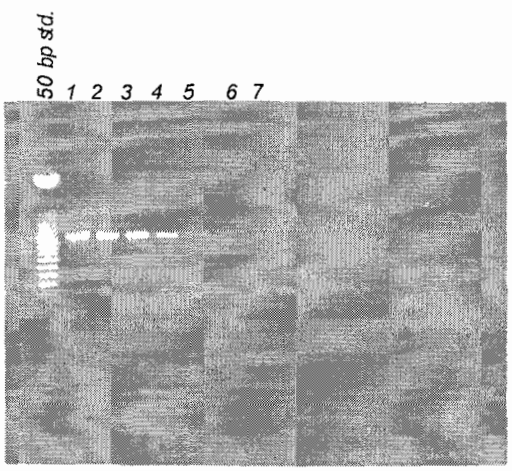

Figure 3:Sensitivity of PM PCR with the KMT, SP$_6$ KMT, T$_7$ primers. PM PCR was performed with DNA extracted from broth culture of serotype A, 10-fold dilution (10^{-1} 10^{-7}); Lanes 1 7. M, 50 bp marker (Roche)

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