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Phenotypic and Genotypic Characterization of *Pasteurella multocida* Isolated from Dead Poultry in Jos, Plateau State.

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SUMMARY

Pasteurella multocida causes fowl cholera in domestic poultry and pasteurellosis in other avian species. This study was carried out due to paucity of information on the isolation and characterization of the organism from poultry in Nigeria. In the present study, 512 poultry were cultured at necropsy for the organism from the liver, spleen, lungs and heart blood, out of which only 6 (1.2%) were positive for *P. multocida*. When the 6 isolates were tested for susceptibility to 15 antimicrobial agents by the standardized single disk method and minimal inhibitory concentrations, all isolates showed multi-drug resistance, but were all found to be susceptible to ciprofloxacin, levofloxacin, cefotaxime, ampicillin-clavulanic acid and imipenem. Furthermore, the isolates were confirmed by species-specific polymerase chain reaction (PCR) and to belong to capsular serogroup B by multiplex PCR. They were also found to harbor plasmids of various sizes. The study established that *P. multocida* may be involved in poultry morbidity and mortality in Jos, Plateau state. The detection of multi-drug resistance among the strains calls for more prudent use of antimicrobial agents and for control through vaccination.

Key words: *Pasteurella multocida*, fowl cholera, biochemical characterization, Microbact 24E, antimicrobial susceptibility, minimal inhibitory concentrations, PCR, multiplex PCR, plasmids.

INTRODUCTION

Pasteurella multocida is an aerobic, gram negative non-motile bacterium that causes fowl cholera in domestic poultry and avian pasteurellosis in other avian species (Amonsin et al., 2002). Fowl cholera is usually seen either as an acute septicaemia with high morbidity and mortality or chronic infections often localized to the joints and sinuses. The high morbidity and mortality associated with fowl cholera result in significant economic losses to the poultry industry (Ali et al., 2004). *P. multocida* belongs to five capsular serogroups, A, B, D, E and F, and 16 somatic serotypes designated 1 through 16 (Rimler et al., 1998; OIE, 2008).

Primary isolation of *P. multocida* is usually accomplished using media such as blood agar, trypticase-Soy agar or dextrose starch agar and isolation may be improved by supplementation with 5% heat-inactivated serum. Discrete, circular, convex, translucent and butyraceous 1 to 3 mm colonies are observed after 18-24 h of incubation. The organism is usually isolated from visceral organs such as liver, bone marrow, spleen, lungs or heart blood of birds that have succumbed to the acute form of the disease, (Townsend et al., 1998; OIE, 2008).

Traditional epidemiologic investigation of fowl cholera have relied largely on biochemical profiling, antimicrobial sensitivity testing, and serotyping. *P. multocida* is a heterogeneous species and considerable variability exists within the taxon (Amonsin et al., 2002;

Petersen et al., 2001; Davies et al., 2004; Ekundayo et al., 2008). But a small proportion of infections in poultry and other animals are caused by the same or closely related strains of *P. multocida* (Davies et al., 2004).

Ekundayo et al. (2008) characterized *P. multocida* strains isolated from avian and other host species in Nigeria. Ambali et al.(2003) reported the occurrence of pasteurellosis due to *P. multocida* as one of the two most important poultry disease in north eastern Nigeria. Episodic outbreaks of pasteurellosis caused by *P. multocida* A:4 in Japanese quail has also been reported in Nigeria (Odugbo et al., 20040.)

Various molecular and genetic methods have been used for identification and epidemiologic investigations of *P. multocida* (Wilson et al., 1993; Townsend et al., 1998; 2001; Petersen et al., 2001; Amonsin et al., 2002; Davies et al., 2004; Kumar et al., 2004; Jabbari et al., 2005; jaglic et al., 2005; Tang et al., 2009). The complete genome of two strains of *Pasteurella multocida* have been determined (Ahir et al., 2011).

Raji et al. (2010) carried out a retrospective study of fowl cholera in Zaria, Nigeria. They found a prevalence of the disease among poultry cases over a period of 5 years to range from 1.5% to 7.4%, with an overall prevalence of 4.7%. Because of the paucity of reports on avian pasteurellosis, they recommended that a national surveillance be conducted to ascertain its endemicity in the country.

To our knowledge, there is only one report on the use of molecular identification of *P. multocida* in Nigeria (Yakubu et al., 2006). In that report, *P. multocida* specific PCR was used to detect the presence of the species in 26% of 54 clinical samples of liver, spleen and cloacal swabs.

Poultry production is increasingly becoming an important source of animal protein in Nigeria. Economic losses due to infectious diseases is the major constraint to the industry due to poor implementation of disease control and prevention measures (Ambali et al., 2003).

This study was carried out to isolate, identify and characterize *P. multocida* from poultry at necropsy in Jos, Plateau State using phenotypic and genotypic techniques.

MATERIALS and METHODS

Sample Collection and Processing

The samples for the study were collected from the Veterinary Hospital, Jos, ECWA Veterinary Clinic, Bukuru and Central Veterinary Diagnostic Laboratory, NVRI, between January and September 2009. The sampling locations were visited two or three times weekly, and all dead or moribund poultry submitted for necropsy were examined at postmortem, from which liver, spleen and lungs were aseptically collected into sterile polythene bags. In addition, the heart was also included in some birds. Samples were placed on ice in a Coleman box and transported to the laboratory within one hour.

Upon arrival at the laboratory, the samples were processed immediately. The surface of each sample was seared using flamed spatula and incisions made with sterile forceps and scissors to take a loopful of the parenchyma or ventricular blood in the case of heart.

The inocula were streaked on either trypticase soya agar, 5% sheep blood agar or Casein Sucrose Yeast extract agar, depending on which agar was available at the time of plating. The plates were incubated at 37^{O}C for about 24 hours.

Biochemical Identification of *Pasteurella multocida.*

Organisms appearing as *P. multocida* on the plating media with discrete, circular, convex, translucent and butyraceous 1 to 3 mm colonies were picked and subjected to gram staining, bipolar staining with methylene blue, indole production and oxidase test.

Organisms that were gram negative, bipolar

staining, indole and oxidase positive were preliminarily considered to be *P. multocida*. Detailed biochemical testing was carried out based on the protocols of Cowan and Steel (1974). The tests included H_2S production in TSI, growth on MacConkey agar, motility, utilization of Simmons citrate, urease production, MR-VP, and fermentation of lactose, sucrose, mannitol, maltose, raffinose, salicin, inositol, trehalose, dulcitol, adonitol, rhamnose, dextrose, fructose, galactose, sorbitol and arabinose. The ability of the isolates to haemolyse red blood cells on blood agar was also determined.

Confirmation of *P. multocida* by Microbact 24E System.

Confirmatory biochemical identification of the isolates was undertaken using Microbact 24E gram negative identification kit (Oxoid Ltd, Basingstoke, UK). The kit uses solid microplate format containing 24 substrates, which was carried out and interpreted after 48 hours based on the recommendations of the manufacturer, using the Microbact software.

Antimicrobial Susceptibility Testing and Determination of the Minimal Inhibitory Concentrations (M.I.C) of Various Antimicrobial Agents Against the Isolates.

The sensitivity of the isolates to 15 antimicrobial agents (Oxoid) was carried out using the standardized single disk diffusion method (Bauer et al., 1966), on 5% horse serum supplemented Mueller Hinton agar, as recommended by the manufacturer and Clinical Laboratory Standards Institute (CLSI, formerly NCCLS). The agents and their disk concentrations are: erythromycin (5μg), tetracycline (30μg), oxacillin (10μg), sulphamethoxazole/trimethoprim (25μg), chloramphenicol (10μg), neomycin (10μg), penicillin (10U), polymyxin B (300μg), vancomycin (5μg), methicillin (10μg), (2μg), gentamicin (10μg), streptomycin (10μg), cloxacillin (5μ g) clindamycin (2μg) and lincomycin (10μg). Briefly, MIC was determined using the MIC evaluator system and interpreted as recommended by the manufacturer to determine the values ranging from 0.015 to < 256 μg/ml (Oxoid).

Determination of Somatic Serotypes of isolates.

The isolates were serotyped at the National Veterinary Services Laboratories, Ames, Iowa using a panel of 16 reference antibodies against Hendelson reference serotypes 1 to 16. Antigen and antisera controls were used in each test.

Identification of *P. multocida* with species specific PCR

Genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniprepTM ZRD6005 (Zymo RT Research, CA, USA). PCR was carried out using primers and cycling parameters reported by Townsend et al., 1998) (Table 1). Briefly, the reaction was carried out in a total volume of 25μl containing 1X PCR buffer, 200μM of each dNTP, 2mM $MgCl_2$, 3.2 pMol of each primer and 0.5 U of Taq DNA polymerase. The reaction was subjected to an initial denaturation at 95°C for 4 minutes and thirty cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for I min, with a final extension for 7 minutes. The reaction was held at 4°C until required for electrophoresis on 2% agarose gel in1X TAE buffer, stained with 1% EtBr, visualized on a UV transluminator and photographed.

***Pasteurella multocida* Multiplex Capsular PCR Typing.**

Multiplex PCR using 5 primer sets for capsular serogroups A, B, D, E, F and species specific pair was carried out as previously reported (Townsend et al., 2001). Briefly the 25 μl reaction contained 1X PCR buffer, 200μM of each dNTP, 2mM $MgCl_2$, 3.2 pMol of each primer, and 1U of *Taq* DNA polymerase. Cycling parameter consisted of initial denaturation of 95°C, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds and a final extension of

72°C for 5 minutes. Agarose electrophoresis and documentation was carried out as described above.

Plasmid DNA Detection.
Plasmid DNA was extracted from 1 ml aliquot of overnight growth of the isolates in brain heart infusion (BHI) broth at 37°C, using a kit and an extraction protocol described by the manufacturer (Fermentas Life Sciences, Maryland, USA).

RESULTS

Isolation and Identification of *Pasteurella multocida*
A total of six (6) isolates of *P. multocida* were obtained from 512 poultry examined during the study, which represents an isolation rate of 1.2%. Of the 6 isolates, 4 were recovered from heart blood and one each from the liver and lungs. The serotypes of the strains could not be detected by the National Veterinary Services Laboratory, Ames, Iowa against the Hendelston reference serotypes. The percentage isolation was much higher from heart blood (2.7%) from 147 samples tested, compared to only one isolate each from liver and lungs and none from spleen in 512 samples examined, thus representing isolation rates of 0.2%, 0.2% and 0.0% respectively. All the isolates were confirmed using the Microbact 2424E system. Although biochemical characterization is the standard for bacterial identification, the isolates gave consistent results only in 13 out of 24 biochemical tests. The Microbact test results were only accepted from the cut-off score of 75% probability as recommended by the manufacturer (Oxoid).

Antimicrobial Susceptibility Testing.
When tested using the single disk diffusion method, all isolates (100%) were resistant to erythromycin, oxacillin, penicillin, metronidazole, clindamycin, cloxacillin, anmd lincomycin, but all were susceptible to neomycin. The other agents to which the bacteria were susceptible in decreasing order were gentamicin (67%), and chloramphenicol (50%) (Table2). The 6 bacterial isolates each displayed different antibiograms (Table3). Two isolates were resistant to 9 agents, one each to 10 and 12 agents, and two isolates displayed resistance to 13 antimicrobial agents. Each of the 6 different patterns in Table3, displayed resistance to 7 common agents.
The M.I.C of 15 antibacterial drugs to the same *P. multocida* isolates were also determined using the MIC Evaluator (Oxoid). Based on established susceptibility break points they were also susceptible to ciprofloxacin, levofloxacin, cefotaxime, ampicillin-clavulanic and imipenem; and resistant to oxacillin, metronidazole, linezolin, penicillin and vancomycin. The overall results of MIC testing and MIC ranges for each agent are given in Table 4. The bacterial isolates were tested to 6 common antimicrobial agents by both the MIC and disk diffusion methods. There was complete agreement in the susceptibility results to penicillin, gentamicin, tetracycline, and oxacillin, and 75% concordance to erythromycin and vancomycin.

Molecular Characterization.
All 6 isolates yielded the 500bp *P. multocida* specific amplicon by PCR, including the Institutes's reference strain used for vaccine production, but not the negative control (Fig. 1). They also yielded a ca. 700bp fragment by multiples PCR consisting of 5 primer pairs for five known capsular serogroups A, B, D, E, and F, suggesting that all isolates belong to capsular group A (Fig. 2). The *P. multocida* specific amplicon, included as internal positive control was also evident. We also demonstrated carriage of plasmids of various molecular sizes by the poultry isolates, all of which harboured a plasmid of about 5 Kb. In addition, 3 isolates were found to have an additional plasmid of 3 Kb.

DISCUSSION

Pasteurellosis or fowl cholera is a common disease of poultry, occurring worldwide, caused

by specific capsular and somatic serogroups and it is also established that distribution and prevalence of serotypes can vary from one region to another (Kumar et al., 2004). Research has shown that fowl cholera is a significant disease of poultry in Nigeria (Ambali et al., 2003; Raji et al., 2010). Most previous studies were largely based on retrospective data, and to our knowledge studies based on isolation, phenotypic and genotypic characterization of isolates have not been undertaken.

Our results showed that the isolates identified in the present study could not be assigned to any of the extant 16 somatic serotypes (Hendelston et al., 1972). The reason for this finding is not clear, although they could belong to new serotypes. However, previous characterization of avian isolates of *P. multocida* revealed a large proportion of untypeable strains (Kumar et al., 2004). But the findings are significant in establishing the occurrence of the aetiological agent of the disease in the study area. The bacterial agents were confirmed by *P. multocida*-specific PCR (Townsend et al., 1998) and belong to capsular serogroup A (Townsend et al., 2001). The later findings are in consonance with the fact that capsular serogroup A is the predominant group infecting poultry (Kumar et al., 2004; Jaglic et al., 2005).

Treatment of fowl cholera usually includes broad spectrum antimicrobials (Rimler and Glisson, 1997). Based on our in vitro antimicrobial testing, the cephalosporins, fluoroquinolones, imipenem and neomycin were the most active drugs. This is in agreement with previous studies on isolates from poultry and pigs (Tang et al., 2009). From our results, the isolates were moderately susceptible to aminoglycosides, but broadly resistant to penicillins, macrolides, and glycopeptides (Tables 1&2). The prevalence of resistance to conventional antibiotics including lincomycin, clindamycin, ttetracycline, trimethoprim-sulphamethoxazole was found to range from 50-100%. Overall, multiple drug resistance (9 to 13 drugs) was found among all the isolates. Although the number of strains tested were few, this is not only disturbing, but may herald the exacerbation of misuse of antibiotics in the country. This may presumably be due to either the uncontrolled use of antibiotic additives in poultry feeds or the extensive use of antibiotics in veterinary medicine. The increased use of antibiotics in poultry production is generally attributed to failure in implementing biosecurity measures. The use of antibiotics in feed is also thought to improve feed conversion and weight gain. It would therefore be advised that antibiotics would have to be carefully selected to be effective for the clinical treatment of fowl cholera or other diseases due to *P. multocida*. It also means that a large pool of resistance genes exist within the microbial communities of poultry population, which could spread to other pathogens including those of zoonotic potentials (Tang et al., 2009).

The current study may be further evidence that *P. multocida* harbour plasmids of various sizes. The roles of these plasmids in bacterial virulence have been suggested (Hunt et al., 2000), but more detailed studies in this regard are warranted. Tang et al. (2009) observed that the distribution pattern of virulence factors in *P. multocida* of swine origin suggests that it is likely that the acquisition of some of them may have led to divergent patterns of vertical and horizontal transmission within the bacterial population through plasmids and other mobile genetic elements.

In conclusion, this study has established that *P. multocida* may be involved in morbidity and mortality in poultry in Plateau state. We also showed that the heart blood may be the preferred organ for the isolation of the organism at post-mortem relative to liver, spleen and lungs, given the fact that most isolates were obtained from this site. It is not known whether the preponderance of untypeable isolates is due lack of available test antisera. Further studies may be required in this direction and to

establish the pathogenicity of the isolates. The fact that all the strains from poultry at necropsy were multi-drug resistant points to the need for more prudent use of antimicrobial agents and for control through vaccination. Further studies are needed to determine the serotypes that are dominant in the country and whether the existing vaccine strain confers protection against endemic serotypes. There is also the need to determine the virulence factors of local strains and role of the plasmids they carry.

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Table1: Primer pairs used for Identification and Capsular Serogroup Determination of *Pasteurella multocida* Isolated from Poultry

Target	Primers	Primer sequence (5′-3′)	Amplicon size (bp)
All species	KMT17	ATCCGCTATTTACCCAGTGG	460
	KMT1SP6	GCTGTAAACGAACTCGCCAC	
Seroproup A	CAP A (F)	TGCCAAATCGCAGTCAG	1044
	CAP A (R)	TTGCCATCATTGTCAGTG	
Serogroup B	CAPB (F)	CATTTATCCAAGCTCCACC	760
	CAPB (R)	GCCCGAGAGTTTCAATCC	
Serogroup D	CAPD (F)	TTACAAAAAGACTAGGAGCCC	657
	CAPD (R)	CATCTACCCACTCAACCATATCAG	
Serogroup E	CAPE (F)	TCCGCAGAAAATTATTGACTC	511
	(CAPE (R)	GCTTGCTGCTTGATTTTGTC	
Serogroup F	CAPF (F)	AATCGGAGAACGCAGAAATCAG	851
	CAPF (R)	TTCCGCCGTCAATTACTCTG	

Table 2: Susceptibility of 6 *Pasteurella multocida* to 15 Antimicrobial agents.

S/No.	Antimicrobial Agents	% Susceptible
1	Erythromycin (E)	0.00
2.	Tetracycline (TE)	33.00
3.	Oxacillin (OX)	0.00
4.	Trimethoprim/Sulfamethoxazole (SXT)	50.00
5.	Chloramphenicol (C)	50.00
6.	Neomycin (N)	100.00
7.	Penicillin (P)	0.00
8.	Polymyxin B (PB)	17.00
9.	Vancomycin (VA)	33.00
10.	Methicillin (MET)	0.00
11.	Clindamycin (DA)	0.00
12.	Cloxacillin (OB)	0.00
13.	Gentamicin (CN)	67.00
14.	Streptomycin (S)	50.00
15.	Lincomycin (MY)	0.00

Table 3: Antibiograms of 6 *P. multocida* tested against 15 antimicrobial agents

S/No.	Isolate	Antibiogram*	No. of Antibiotics
1	EC 243H	E,OX,C,P,VA,MET,DA,OB,MY	9
2	EC 251H	E,OX,P,PB,VA,MET,DA,OB,MY	9
3	EC 297H	E,TE,OX,SXT,C,P,PB,MET,S, CN, DA,OB,MY	13
4	EC 238V	E,TE,OX,SXT,C, P,PB,VA,MET,DA,OB,MY	12
5	EC 292U	E,TE,OX,SXT,P,PB,VA,MET,DA,OB,CN,S,MY	13
6	EC 261H	E,TE,OX,P,PB,MET,DA,OB,S,MY	10

* See Table 2 for the identity of antimicrobial agents

Table 4: Minimum Inhibition Concentration (MIC) Ranges of 15 Antimicrobial Agents Against 6 *P. multocida* Isolates

S/No.	Antimicrobial Agent	% Strains Susceptible	M.I.C Range (µg)
1.	Erythromycin (E)	33.00	6->256
2.	Oxacillin (OX)	0.00	24->256
3.	Cefotaxime (CTX)	100.00	0.03-0.12
4.	Ampicillin-Clavulanic acid (AMC)	100.00	0.03-16
5.	Tetracycline (TE)	33.00	1.0-128
6.	Metronidazole (MTZ)	0.00	>256
7.	Ampicilin (AMP)	67.00	0.37->256
8.	Gentamicin (CN)	67.00	0.06-48
9.	Levofloxacin (LEV)	100.00	0.06-6.0
10.	Linezolin (LZD)	0.00	>256
11.	Amikacin (AML)	67.00	0.25->256
12.	Imipenem (IPM)	100.00	0.023-0.12
13.	Penicillin (P)	0.00	16-256
14.	Ciprofloxacin (CIP)	100.00	0.004-2.0
15.	Vancomycin (VA)	0.00	48->256

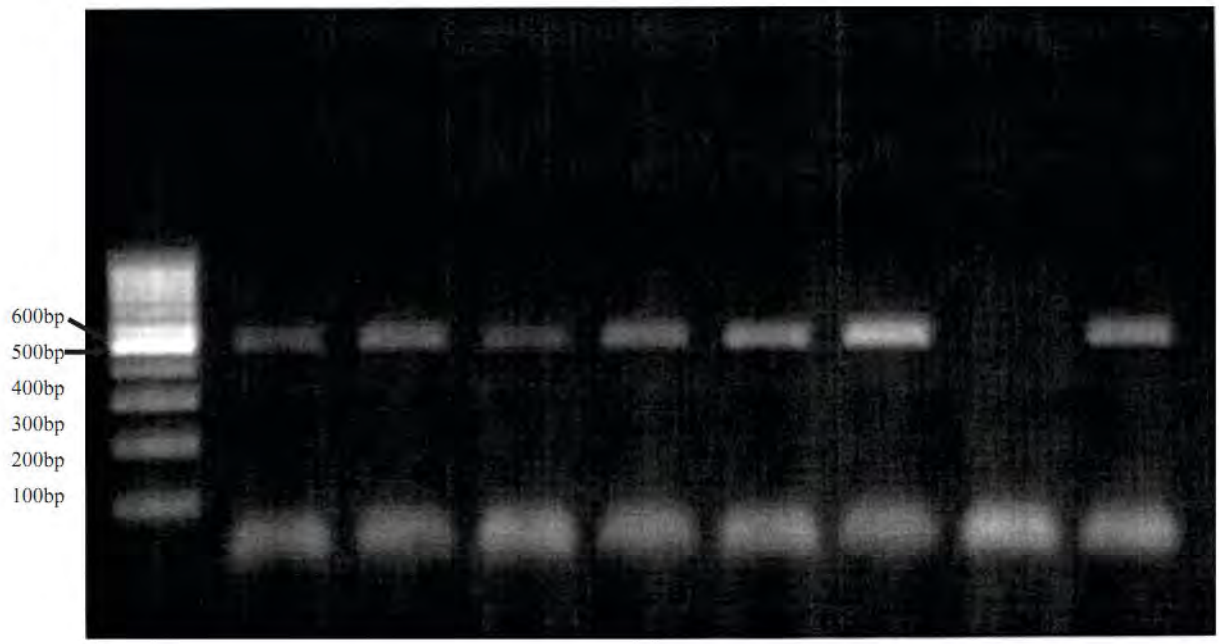

Figure 1: Identification of *P. multocida* by species specific Polymerase chain reaction.
Lanes 1 to 6 are the avian isolates, lane 7 negative control and lane 8 positive control. Molecular size markers, 100 bp ladder (Inqaba) is in lane M.

Figure 2: Multiplex typing of the capsular serogroups of *P. multocida*.The 6 test isolates are included in lanes 1-6, while lane 7 is the positive control. Lane M contains the molecular size markers.

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