Characterization and Serotyping of *Pasteurella multocida* isolated from Deep Litter and Free-range Chickens in Vom, Plateau State, Nigeria

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Abstract: Fowl cholera caused by *Pasteurella multocida* is a signifant threat to the poultry industry that hampers profit margin for poultry production. This study was conducted to investigate the prevalence and serotypes of *P. multocida* among deep litter and free-range chickens in Vom, Nigeria. A total of 200 cloacal swabs, comprising of 100 each from healthy deep litter and free-range chickens were aseptically collected for the isolation of *P. multocida* by standard isolating procedures. Biotyping, capsular and somatic serotyping of the isolates was carried out by sugar fermentation test, hyaluronic acid test and agar gel diffusion precipitin test respectively. The prevalence of *P. multocida* in deep litter and free-range chickens was 7% and 4% respectively. All the isolates from deep litter and free-range were *P. multocida* as confirmed by the detection of KMT1 sequence using PM-PCR and were subsp. *multocida* and subsp. *septica* by sugar fermentation test. Capsular type A and somatic type 4 were detected among 6 out of 7 from deep litter isolates, while one remained untypeable using both the capsular and the somatic typing methods. All the isolates (n=4) from free-range chickens remained untypeable by both methods used in this study. This study elucidates the importance of periodic epidemiological survey for *P. multocida* in different chicken management system in other to understand the nature of the causative organism in terms of capsular and somatic types so that it may be incorporated in the fowl cholera vaccine production

Keywords: *Pasteurella multocida*, chicken, fowl cholera, cloacal swab, biotyping, serogrouping

INTRODUCTION

Poultry, especially chickens, play an essential role in the lives and livelihoods of millions of rural and urban households worldwide. Poultry production in rural areas is considered a primary source of eggs and meat (Panna *et al.,* 2015). However, despite the enormous benefits derived from birds, the poultry industry is faced with enormous challenges of emerging and re-emerging diseases of both public health and socioeconomic importance (Shivachandra *et al*., 2005).

Amongst the numerous diseases affecting chickens, Fowl cholera, also known as avian Pasteurellosis or avian hemorrhagic septicemia, constitutes a significant burden to the survival of this poultry industry, especially in Sub-Saharan Africa (Wang *et al*., 2009). It is a disease of domesticated and wild birds, which is a devastating and killer disease. The condition causes severe economic loss and hampers the development of the poultry industry in developing countries (Ievy *et al.,* 2013).

Fowl cholera (FC) occurs sporadically or enzootically in most countries of the world (Ievy *et al.,* 2013) and the incubation period ranges from four to nine days, but acute outbreaks can occur within two days of infection. Most outbreaks of fowl cholera affect chickens, turkeys, ducks and geese. Fowl cholera is caused mainly by *Pasteurella multocida*, a capsular, Gram negative, non-motile, non-spore-forming facultative anaerobic bacillus and a member of the family Pasteurellaceae (Wang *et al*., 2009; Xiao *et al*., 2016). *P. multocida* has three subspecies namely: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica* (Dziva *et al.,* 2008).

P. multocida is often associated with severe economic loss in poultry due to the loss of cattle or poultry species (Marza *et al*., 2015). The FC, a septicemic disease, is associated with high morbidity and mortality in poultry, especially chicken and ducks (Akhtar *et al.,* 2016).

Identification and differentiation of pathogenic bacteria strains are the two primary requisites for epidemiological studies. The phenotypic methods, like serotyping and biotyping, have been used to differentiate the strains (Shirzad-Aski and Tabatabaei, 2016). Nucleic acid-based assays have improved bacterial detection and identification. Genetic techniques such as PCR are routinely used in veterinary medicine to diagnose infectious diseases such as fowl cholera and play an essential role in the clinical laboratory as rapid and specific detection of micro-organism (Townsend *et al*., 2001).

Pasteurella multocida serotyping depends on typing systems mainly capsular typing using hyaluronic acid test and somatic typing techniques using agar gel diffusion precipitin test (Abbas *et al.,* 2018). *Pasteurella multocida* belongs to five capsular serogroups (A, B, D, E and F) and sixteen somatic serotypes (1-16) (Kwaga *et al.,* 2013).

P. multocida is the major cause of respiratory tract infection in poultry and is harboured in the cloacal mucosa of asymptomatic birds and these strains remain as sources of outbreaks (Ievy *et al.,* 2013). Pathogenicity or virulence of *P. multocida* is variable and complex, depending on the host species, strain, variation within the strain or host, and conditions of contact between two birds (Panna *et al.,* 2015).

Control of *P. multocida* in poultry could be achieved with a vaccine-based approach. However, the protective efficacy of *P. multocida* vaccines is variable, particularly against heterologous strains (Massacci *et al*., 2018). In Nigeria, locally produced and foreign vaccines have been used over the years as a preventive measure to curb the outbreaks of Pasteurellosis. However, with the continued rise in the cases of FC in most parts of the country, which could be attributed to the serotype-specific nature of the immune response by naïve chickens, there is the need for the isolation and characterization of field isolates of *P. multocida* circulating in Nigeria in a bid for the production of effective vaccines. Therefore, the present study aimed to characterize the *P. multocida* isolates from a deep litter and free-range chickens in Vom, Plateau State, Nigeria.

MATERIALS AND METHODS

Collection of samples

For this study, a total of 200 cloacal swab samples were aseptically collected using non-toxic sterile swab sticks from healthy deep Litter (*n*=100) and free-range (*n*=100) chickens in some selected backyard farms and households in Vom, Plateau state, Nigeria. All the samples were correctly labelled and transported to the laboratory in a cold ice-pack container.

Isolation and characterization of *Pasteurella multocida*

The cloacal swabs were inoculated into Brain heart infusion broth containing 5µg/mL of lincomycin in Bijou bottle, then diluted serially in the same type of medium from 10^{-1} to 10^{-5} and incubated overnight at 37^{o}C. The highest dilution showing turbidity was inoculated onto 5% sheep blood agar and incubated at 37^{o}C for 24 h (Pijoan *et al*., 1983). Colonies with characteristic cultural morphology of *Pasteurella* were further identified based on their Gram morphology and biochemical reactions. The isolates were typed into subspecies using sugar fermentation tests.

PCR further confirmed all the isolates that were suggestive of *Pasteurella multocida* were further confirmed by PCR, this is performed by first extracting the genomic DNA as described by Silhavy *et al.* (1984) and *Pasteurella multocida* specific PCR was performed according to the method of Townsend *et al.* (1998). The forward and the reverse primers 5-GCTGTAAACGAACTCGCCAC-3 and 5-ATCCGCTATTTACCCAGTGG-3. The PCR reaction and the cycling procedure were done according to Townsend *et al.* (1998). PCR products were resolved on 1.5% agarose containing 10µl of 10mg/ml ethidium bromide at 60-80 volts for 60 minutes. One hundred base pair marker (Roche, Mannheim, Germany) was used as molecular ladder. A gel documentation system was used to visualize the product.

Serotyping of *Pasteurella multocida*

Capsular serotyping of the *P. multocida* isolate was carried out by hyaluronic acid test while somatic serotyping was carried out by agar gel precipitation test as described by Carter (1984).

Statistical analysis

Prevalence of *P. multocida* isolated from deep litter and free-range chickens were compared using Chi-square test with the aid of the GraphPad Prism software (version 6.0). Values of $P<0.05$ were considered statistically significant for this study.

RESULTS

The colonial morphology, microscopic and biochemical characteristics of the isolates is shown in Table 1. *P. multocida* isolates appeared as small, mucoid and non-haemolytic colonies on 5% sheep blood agar. Microscopically the isolates were Gram negative short rods and biochemically they gave A/A reaction on TSI agar, were H_2S, catalase, oxidase and indole positive, Urease negative with no visible growth on MacConkey agar.

In this study, 11 *P. multocida* were isolated from the 200 cloacal swabs collected, representing 5.5%. Out of the 11 isolates, 7 and 4 were isolated from the deep litter and free-range chickens, representing 7.0% and 4.0% respectively. There was no significant difference in the number of *P. multocida* isolated between the two rearing systems (p= 0.35212) (Table 2).

Based on the result of biotyping by sugar fermentation, all the isolates from the deep litter chickens were *P. multocida* subsp *multocida*. In contrast, all the isolates from the free-range chickens were *P. multocida* subsp *septica* (Table 3).

Table 1: Characterization of *Pasteurella multocida* isolated from Deep Litter and Free-range chickens in Vom, Nigeria

Isolate code	Colonial morphology on BA	Gram reaction	TSI	H_2S	Ind	Cat	Oxi	Ure	Growth on MCA	Inference
DL01	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL02	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL03	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL04	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL05	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL06	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
DL07	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
FR01	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
FR02	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
FR03	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*
FR04	Small, mucoid and non-haemolytic	Gram negative short rods	A/A	+	+	+	+	-	-	*Pasteurella multocida*

Key: DL = isolates from deep litter chicken, FR = isolates from free-range chickens, A = acid, TSI = sugar fermentation on Triple Sugar Iron Agar, ind = indole test, cat = catalase test, oxi = oxidae test, ure = urease test, MCA = MacConkey agar, + = positive, - = negative

Table 2: Prevalence of *Pasteurella multocida* isolated from the cloaca of deep-litter and free-range chickens in Vom, Nigeria.

Rearing System	No. of cloacal swabs examined	No. *P. multocida* isolated (%)
Deep Litter	100	7 (7.0)
Free-range	100	4(4.0)
Total	**200**	**11 (5.5)**

p= 0.35212

The serotypes of the *Pasteurella multocida* isolated from deep litter and free-range chickens is presented in Table 4. Six out of the seven isolates from the deep litter chicken were found to belong to the capsular type A and somatic type 4 serotype however the remaining one isolate was untypeable by both capsular and somatic typing. All the isolates from free-range chickens were untypeable by both capsular and somatic typing. Plate I shows result of the hyaluronic acid test for capsular serotyping while Plate II shows result of agar gel precipitin test for somatic typing.

Table 3: Biotyping of *Pasteurella multocida* isolated from Deep Litter and Free-range chickens in Vom, Nigeria using sugar fermentation

Isolate code	Glucose	Maltose	Sorbitol	Dulcitol	Trehalose	Xylose	Arabinose	Inference
DL01	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL02	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL03	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL04	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL05	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL06	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
DL07	+	-	+	-	+	-	-	*P. multocida* subsp. *multocida*
FR01	+	-	-	-	+	+	-	*P. multocida* subsp. *multocida*
FR02	+	-	-	-	+	+	-	*P. multocida* subsp. *multocida*
FR03	+	-	-	-	+	+	-	*P. multocida* subsp. *multocida*
FR04	+	-	-	-	+	+	-	*P. multocida* subsp. *multocida*

+ = acid from sugar fermentation, - = no acid from sugar fermentation, PM = *Pasteurella multocida* subsp. *multocida*

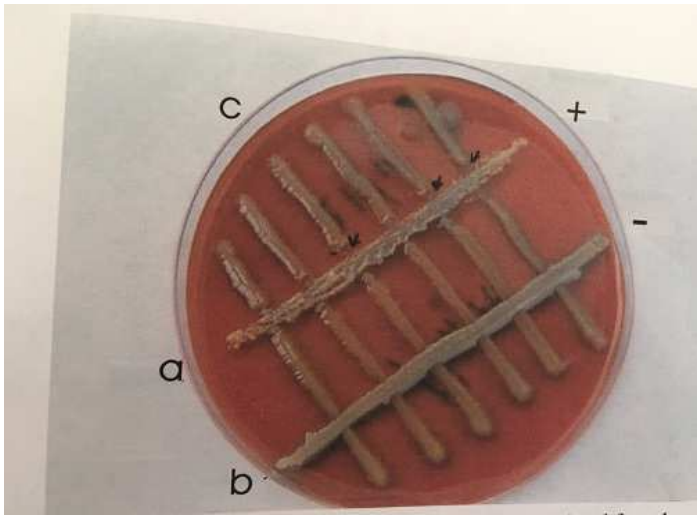

Plate I: Hyaluronic acid test result of *Pasteurella multocida* isolated from Deep Litter and Free-range chickens in Vom, Nigeria. **a** = Horizontal streak of *P. multocida*, type B producer of hyaluronidase; **b** = Horizontal streak of *Escherichia coli*, non-producer of hyaluronidase; **c** = Vertical streaks of test strains of *P. multocida* Type A, producers of hyaluronic acid; + = Positive; - = Negative. Arrows on horizontal streak indicate hyaluronidase activity seen as no growth zone.

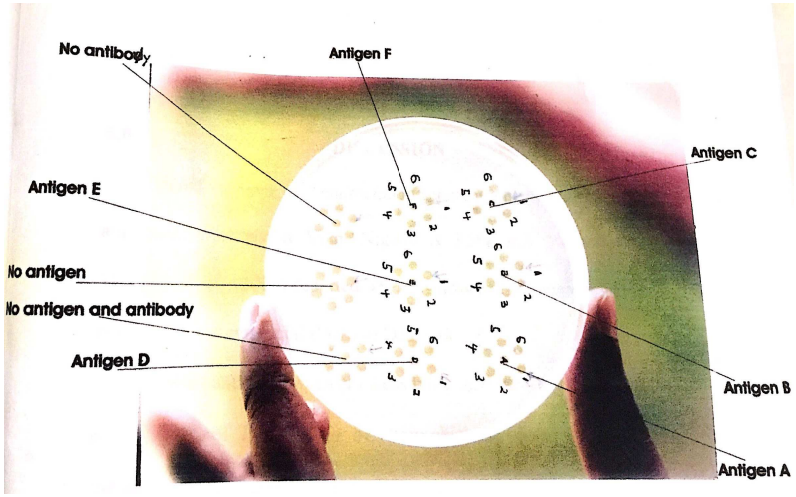

Plate II: Agar Gel Precipitin test of *Pasteurella multocida* isolated from Deep Litter and Free-range chickens in Vom, Nigeria. 1-6 are numbers of somatic antisera, A-F are *P. multocida* antigen.

Table 4: Serotyping of *Pasteurella multocida* isolated from deep litter and free-range chickens

Isolate code	Source of isolate	Capsular type	Somatic type
DL01	Deep litter chickens	A	4
DL02	Deep litter chickens	A	4
DL03	Deep litter chickens	A	4
DL04	Deep litter chickens	A	4
DL05	Deep litter chickens	UT	UT
DL06	Deep litter chickens	A	4
DL07	Deep litter chickens	A	4
FR01	Free-range chickens	UT	UT
FR02	Free-range chickens	UT	UT
FR03	Free-range chickens	UT	UT
FR04	Free-range chickens	UT	UT

Key: UT = untypeable

DISCUSSION

Pasteurella multocida has a worldwide distribution and is considered part of the normal respiratory microbiota of many animals. However, it is potentially pathogenic in domestic and agricultural animals (Wilkie *et al*., 2012; García-Alvarez *et al*., 2015). The overall prevalence of *P. multocida* in chickens in Vom was found to be 5.5%. Isolation of *P. multocida* from the cloaca of chickens in this study further shows that the cloaca may plays a significant role in the transmission dynamics of *P. multocida* species in chickens. This is in agreement with the findings of Muhairwa *et al*. (2001).

Higher prevalence of *P. multocida* were reported by previous researchers: 12.05% was reported by Hasan *et al.* (2010), 59.72% was reported by Belal (2013) in backyard poultry, 13.04% was reported by Hossain *et al.* (2013) while Panna *et al.* (2015) reported 11.42% in chickens. The difference observed may be due to differences in breed, management system, age and resistance status due to improved management, vaccine and nutrition.

The prevalence of *P. multocida* in this present study was found to be higher in deep litter chickens (7.0%) than free range chickens (4.0%). This is likely due to sharing of common points of feeding and drinking in deep litter management system since the most common portal of entry for *P. multocida* is the nasal inlet. So also, close contacts between chickens in commercial flocks have been shown to enhance the transmission of *P. multocida* (Muhairwa *et al*., 2001).

Pasteurella multocida subsp *multocida* was the predominant subspecies in this study, this finding is in agreement with the findings of Snipes *et al*. (1990) and Townsend *et al*. (2001) who reported that *P. multocida* subsp *multocida* were the predominant subspecies causing fowl cholera in turkeys and chickens.

We also investigated the capsular serotype and our findings revealed serotype A as the only serotype of *P. multocida* observed in this study. This findings agrees with previous outcomes on the serotyping of *P. multocida* that capsular serotype A predominates among avian species as the major cause of Fowl Cholera (Kwaga *et al*., 2013; Bhimani *et al*., 2018).

Similarly, all the serotyped isolates were found to belong to somatic serotype 4. This result is in tandem with the findings from previous studies showing the presence of serotypes 1, 3 and 4 of serotype A as the common etiologies of Fowl Cholera (Hirsh *et al*., 1990).

All the isolates from free-range chicken and one isolate from deep litter chicken were untypeable. Generally, characterization of *P. multocida* isolated from chickens and birds have revealed large proportion of untypeable strains (Kwaga *et al.,* 2013). The presence of untypeable isolates might be attributable to new strains that are not included among the 16 Handlestone reference samples or due to loss of somatic antigens by the isolates (Sugun *et al.,* 2016). Our inability to unveil the type of strains in this study is a limitation and this is due to available techniques within our budget

The eleven isolates that were suggestive of *Pasteurella multocida* by biotyping, each generated a single band at 460bp when measured against 100bp marker (Roche, Germany), this confirmed the isolates as *P. multocida*. The PCR assay is capable of distinguishing *P. multocia* from other *Pasteurella* species and closely related genera (Geraldo *et al*., 2001), but is limited to subspeciation.

CONCLUSION

In conclusion, the present study has further confirmed that the cloaca mucosa is another habitat for *P. mutocida* in live chickens. Though *P. multocida* was more evident in the commercial chickens on deep litter in the study area, the present study has indicated the presence of both *P. multocida* subsp *multicida* and *P. multocida* subsp *septica* in chickens in Vom, Nigeria, suggesting that these serotypes could be included into a multivalent vaccine if the successful prevention and or control of Fowl cholera is to be achieved. We therefore recommend that further pathogenic and molecular epidemiological studies should be conducted on all the capsular strains in the study area so as to understand their roles in the disease manifestations.

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