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Isolation of Uncommon *Pasteurella multocida* Strains from Cattle in North Central Nigeria

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Abstract

The prevalence of *P. multocida*, an aetiologic agent of bovine haemorrhagic septicaemia was studied using a purposive sampling technique in north central Nigeria. A total of 18 positive isolates of *P. multocida* were obtained from 175 lungs, liver, and spleen samples examined, giving an isolation rate of 10.3%. Nineteen isolates were confirmed as *P. multocida* by Microbact GNB 24E supplied software version Microbact™ 200 identification package V2.03 (Windows™) and by species specific PCR. By the software interpretations package the percentage probabilities of 12 isolates were above 75% and 7 others were below 75%. The study confirmed the presence of the African capsular strain E (511 bp) and a unique capsular F type. The *P. multocida* strains were somatically typed as: *P. multocida* E: 3, 4 and *P. multocida* E: 2, 5, but most were untypeable. Also of interest is capsular group F somatically untypeable strain being identified for the first time from calves in Nigeria. These strains have not previously been reported in Nigeria or within the West African sub-region. These could redefine the vaccine strategy as the current vaccine used in Nigeria contain *P. multocida* B: 3,4 and E: 2. However more work needs to be carried out in other parts of the country to gather more relevant information with regards to capsular and somatic types.

Keywords: *Pasteurella multocida;* Animals; PCR; Serotypes

Introduction

The Gram negative bacterium *P. multocida* infects a wide range of animal species, causing diseases such as atrophic rhinitis in pigs [1], fowl cholera in poultry [2] and haemorrhagic septicaemia and shipping fever in cattle [3,4]. First reported type-specific capsular antigen in *P. multocida* [5]. Later, there were five distinct capsular serogroups of *P. multocida* (serogroups A, B, D, E and F) indicated [6]. In addition to the capsule serogroups, 16 major somatic serotypes (Identified as 1 through 16) were recognized. Lipopolysaccharides, determined by gel diffusion precipitin test [6] are the antigens that determine somatic type specificity. Various somatic serotypes occur among the different capsule serogroups and current serological nomenclatures designate both of these features.

It is well known that P. multocida with certain antigenic compositions can be associated with specific diseases in animals. For example P. multocida B:2 and E2 serotypes cause haemorrhagic septicaemia in cattle. However, a substantial reclassification of bovine relevant *Pasteurellaceae* has been carried out since 1999, starting with the introduction of the new *genus* Mannheimia [6]. Consequently, the accuracy of a routine bacterial diagnosis of bovine pasteurellosis, and of the epidemiology and the antimicrobial susceptibilities of Pasteurellaceae based on former investigations is questionable. Thus, there is a need to reinvestigate bovine Pasteurellaceae according to this reclassification assisted by currently indispensable molecular identification tools [7]. Reported a PCR based method for the capsular typing of P. multocida. The capsular PCR is now regarded as the gold standard test [8] and has been used in a number of studies of isolates from a range of animal hosts [9-11]. While the advantage of the PCR based approach has been well recognized, the adoption of PCR-based technologies in Veterinary laboratories in the developing world has been limited in some areas by factors such as cost and technical expertise [12]. Reported that PCR-based typing was more discriminative and could further subtype previously untypeable strains. PCR serotyping is therefore a highly speciesspecific, sensitive and robust method for detection and differentiation of P. multocida serogroups compared to conventional serotyping [13]. Organisms belonging to the bacterial family Pasteurellaceae are ubiquitously present in the respiratory, alimentary and reproductive tracts of different avian, mammalian, reptilian, and likely amphibian hosts [14]. Haemorrhagic septicaemia and mastitis caused by P. multocida is among the important diseases, which in case of mistaken diagnosis can cause a high rate of mortality in cattle [15]. Besides P. multocida types B:2 and E:2 which are the main causes of haemorrhagic septicaemia, many other serotypes viz; B:3, B:4, B:3,4, B:2,5, B:5, E:2,5 have reportedly been encountered in feral and domestic ruminants including cattle, deer, elk and bison [16-18]. The lists of haemorrhagic septicaemia causing strains of P. multocida appear to be widening. There is a dearth of research in this area and this has led to several unresolved questions. For example, besides the so-called African serotype or strain (E: 2), to what extent are new or other serotypes of P. multocida involved in haemorrhagic septicaemia outbreaks in Africa. Also to date a meaningful attempt has not been made to relate the field strains and reference vaccine strains, or for effective control of the disease, which strains need to be represented in the vaccine. Both

documented and anecdotal evidence of outbreaks of haemorrhagic septicaemia in Nigeria and the rest of African continent abound [3,19]. Constant surveillance for current serotypes is therefore imperative to contain bovine haemorrhagic septicaemia. The objective of this study was to determine by means of purposive sampling technique the prevalence of P. multocida within visceral organs of cattle from abattoirs located within nine LGAs of Plateau state, Nigeria (Figures 1 and 2).

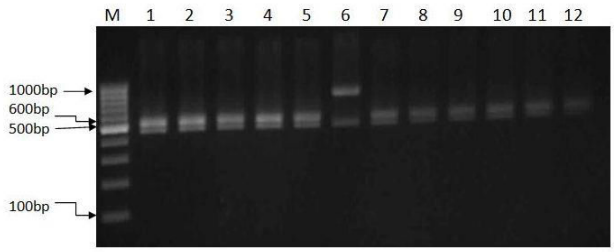

Figure 1: Multiplex PCR profiles with species-specific primers as internal control. Lane 1 (Bld3), Lane 2 (Bld 9) Lane 3 (Bld10), Lane 4 (Jst2), Lane 5 (Js8), lane 6 (Jn3) Lane 7 (Bld3), Lane 8 (Bld3), Lane 9 (Jn6), Lane 10 (Jn12), Lane 11 (Jn14), Lane 12 (Jn18), Lane 12 (Ka2) and and lane M, 100 bp DNA marker.

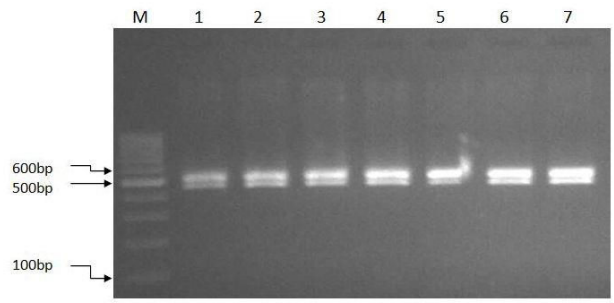

Figure 2: Multiplex PCR profiles with species-specific as internal control Lane 1 (Ka3), Lane 2 (ka4), Lane 3 (ka5), Lane 4 (Mg4), Lane 5 (Mg7), Lane 6 (Ot2), Lane 7 (Jn6) lane M 100 bp DNA molecular size maker.

Materials and Methods

Sampling

A total of one hundred and seventy five samples consisting of lungs, liver and spleen were collected from abattoir/slaughter slabs in nine Local Government Area of Plateau state. The samples were placed in sterile bottles, kept in a Coleman box containing ice and transported to the laboratory for examination.

Isolation of *Pasteurella multocida*

The tissues were cultured directly on casein sucrose yeast agar (CSY) agar, incubated at 37°C for 24 hours [20]. Single non-haemolytic colonies were selected from primary culture and restreaked on fresh Blood agar (BA) plate and incubated at 37°C for 24 hours to obtain single colonies of the isolates. The cultures so obtained were subjected to Gram's staining to check for purity of the growth morphology of the organisms and their ability to grow on MacConkey agar (MCA). The isolates which failed to grow on MCA were preliminarily presumed to be *P. multocida*. The cultures were then transferred onto nutrient agar slants for storage, pending further identification by biochemical tests. Further tests included: Oxidase, catalase, indole, citrate utilization, nitrate reduction and fermentation of glucose, mannitol, sucrose, mannose, maltose, arabinose, lactose, dulcitol, salicin, inositol and trehalose using r methods described by Cowan and Steel with modification of using CSY agar [21].

Identification of *Pasteurella multocida* using Microbact GNB 24E

Identification of *P. multocida* using Microbact GNB 24E (Oxoid, Basingstoke, UK) was employed as a confirmatory test. Before testing, all isolates were streaked on blood agar containing 5% bovine blood and incubated at 37°C for 24 hours. Sterile normal saline was prepared and 5 ml dispensed into each test tube. Using a sterile loop, 1-3 isolated colonies of the culture were picked and emulsified in the 5 ml sterile saline, which was mixed thoroughly and incubated at 37°C for 4 hours. The wells of the individual substrates sets were exposed by cutting the end tag of the sealing strip and slowly peeling backward. The plate was placed in a holding tray and using a sterile Pasteur pipette, four drops of the bacterial suspension were added to each well. Using a sterile pipette, the substrates underlined on the holding tray were overlayed with mineral oil i.e. wells 1, 2, 3, 20 and 24. The plates were incubated at 37°C for 48 hours. The plates were removed from the incubator after 48 hours, the adhesive seal peeled and Nitrate, Kovacs, VP and TDA reagents were added to wells 7, 8, 10 and 12 respectively. The results were interpreted as stipulated by the manufacturers and the organisms identified using the software version Microbact^{TM} 200 identification package V2.03 (Windows^{TM}).

Somatic serotyping of isolates

The isolates were shipped to the American National Veterinary Services Laboratories, USDA, AMES, IOWA, USA for somatic typing: The isolates were typed using the procedure of Somatic serotyping [22]. A panel of 16 reference antibodies made against Henddleston reference serotypes 1-16 was used in the typing procedure. Antigen and antisera controls were used in each test. The isolates were further confirmed by PCR using species specific primers: PCR: KMT1T7 5'-ATC-CGC-TATTTA-CCC-AGT-GG-3' KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'.

Capsular typing via polymerase chain reaction (PCR)

The detection of capsular genes by PCR for all serogroups was done according to the method described by [23]. An overnight cell culture grown in Brain Heart Infusion (BHI) broth was harvested by centrifugation at 11,337$_x$g for 5 min. The cell pellet was resuspended in 100 µl of TE (10 mM Tris; 1 mM EDTA, pH 8.0) buffer and boiled for 10 min, followed by immediate chilling. The cell lysate was centrifuged at 11,337$_x$g for 5 min and the supernatant was used as the DNA template. The concentration of the DNA template was determined using a spectrophotometer at OD 260/280 nm (Eppendorf, Germany). The oligonucleotide sequences of primers previously published were synthesized by Operon (Germany) (Table 1) [24].

S.No	Isolatees No	Specimen	OCT INDEX	% Probability	Somatic serotypes
1	Bld 3	Spleen	515632744	96.23	Untypeable
2	Jn 12	Lung	517632660	92.75	Untypeable
3	Jn 14	Lung	537736600	99.19	Untypeable
4	Jn 18	Lung	526733060	97.56	3,4
5	Ka 2	Lung	517622664	75.4	Untypeable
6	Ka 3	Lung	517732560	93.56	Untypeable
7	Jn 5	Lung	517232720	85.44	2,5
8	Jst2	Liver	517722700	97.14	Untypeable
9	Ot2	Lung	517720700	99.35	Untypeable
10	Bld 9	Liver	517722760	77.46	Untypeable
11	Mg 7	Lung	577737500	91.45	Untypeable
12	Bld 10	gnuL	517722700	97.14	Untypeable
13	Jn 6	Liver	517632764	66.16	Untypeable
14	Jn 3	lung	517231760	69.77	3,4
15	Ka 4	Liver	513622742	56.58	Untypeable
16	Js 8	Lung	517732750	48	Untypeable
17	Ka 5	Liver	517722764	42.96	Untypeable
18	Mg 14	Spleen	517732750	48	Untypeable

Table 1: Percentage of *P. multocida* isolates with Microbact GNB 24E and Somatic.

Results

Biochemical Characteristics of *Pasteurella multocida*

All isolates were gram negative and positive for catalase, indole production, oxidase, citrate utilization and nitrate reduction. All isolates fermented glucose, mannitol, sucrose, mannose and were negative for maltose, arabinose, lactose, inositol and trehalose. No growth was observed on MCA agar. Isolates were confirmed by Microbact 24E, where 12 of the isolates had a probability percent above 75 % and six other isolates had probability percent below 75 % by the software interpretation package. Out of the 19 positive isolates, 61.1 % were from lungs, 27.7 % were from liver and 11.1 % from spleen. Three somatic serotypes (3,4. 3,4. 2,5) were identified among the somatic antigens. All the remaining 15 isolates were reported as un-typeable Table 1. Jos North had the highest percentage positive samples of 13.5 %, followed by Kanam L.G.A with 9%. Barkin ladi had a percentage positive sample of 6.8 %, Jos south 4.5%, Mangu 4.4% and Quanpaan 2.2%. Langtang North, Ryom and Pankshin did not have any positive samples.

Discussion

Pasteurella multocida is an important pathogen causing a number of diseases in various domestic and wild animals and avian species. The most important diseases are haemorrhagic septicaemia and septicaemic pasteurellosis in cattle and buffaloes, pneumonia and septicaemic pasteurellosis in sheep and goats, pneumonia, atrophic rhinitis and septicaemia in pigs and fowl cholera or avian cholera in poultry/turkey resulting in heavy economic losses [25]. However, the distribution and prevalence of *P. multocida* serotypes and pathotypes can vary considerably from region to region and over time in a given region. Contemporary studies aimed at unraveling or defining the current spectrum of *P. multocida* serotypes in cattle in Nigeria are lacking. The overall isolation percentage of *P. multocida* from the total number of samples examined was 10.3 % (Table 1). The various characteristics of *P. multocida* isolated during the present investigation are in accordance with the findings of Francis and Carter who reported the isolation of 17.5% *P. multocida* from calves in Zambia [26]. Variation in the characteristic of *P. multocida* has been reported by different authors; for example [27] found variability in oxidase test. Researchers who collected samples from cattle with frank clinical signs of haemorrhagic septicaemia or directly from lungs with obvious pathological lesions reported high incidence of *P. multocida*, including Kielstein & Schimmel, who reported an isolation rate of 50.4% in 115 calves with pneumonia in ten herds and [27] also reported an incidence of 25.2% in samples collected from lung lesions. The variability observed in this study might be due to geographical variation in prevalence and use of chemotherapeutic agents, which may influence the occurrence. Distribution of serogroups by multiplex PCR was determined. Serogroup E was found in 4.4% samples and serogroup F in 5.6 % samples examined. Isolates were also typed

somatically. A notable feature of the current study was the preponderence of untypeable strains. It is interesting that although untypeable strains are generally uncommon in cattle, 83.3% of isolates were somatically untypeable and only 16.7% were typeable. It is not known whether the presence of untypeable isolates were attributable to new strains that are not included among the 16 Handlestone reference samples or due to loss of somatic antigens by the isolates. During the study period, other bacterial species, including *Haemophilus parasuis* and *Streptococcus suis*, were often co-isolated with pathogenic *P. multocida* strains from the same samples. Although it is not straightforward to distinguish whether *P. multocida* is a primary or a secondary pathogen in mixed infections, the fact that various bacterial species may coexist in a given herd should be considered when attempts are made to control disease outbreaks [28]. *Pasteurella multocida* serotypes B:2 (6:B) and E:2 (6:E) are the principal causes of HS. Although serotype B: has been mainly reported in Asian countries and E:2 in African countries [20-23], both serotypes have been recovered from the disease in some African countries [23]. Beside type B:2, several other B serotypes (B:3, B:4, and B:3,4) have been incriminated in recent years in sporadic outbreaks of HS in cattle and feral ruminants such as deer, elk and bison [11]. In Africa, serotype E:2 appears to be dominant with cattle rather than water buffaloes being mainly involved. In the present study, the African strain E: 2,5; E:3,4 and a unique strain F which somatically was untypeable were encountered.

Conclusion

In conclusion, haemorrhagic septicaemia is a major disease of cattle in Africa caused by *P. multocida* serotypes. The disease has been a long standing problem with outbreaks of the disease occurring an annual basis. Although vaccination of cattle has long been the hub of control programe, haemorrhagic septicaemia which is an OIE- listed disease and second to contagious bovine pleuropneumonia in its devastation of cattle in sub-saharan Africa has not been adequately controlled partly due to available vaccines which are undefined and of variable efficacy. Over the years, reports of haemorrhagic septicaemia in Nigeria have been inconclusive as information on the specific serotypes of *P. multocida* involved is seldom determined. It is also not known whether the so-called untypeable strains are also involved in haemorrhagic septicaemia outbreaks. The present study has evidently shown the existence in Nigeria of hitherto unencountered serotypes of *P. multocida*. The mounting evidence of several more serotypes is a challenge as it has limited our ability to control adequately haemorrhagic septicaemia resulting from infection with *P. multocida*. More epidemiologic studies are however advocated and appropriate comparative transmission studies are underway to determine the pathogenicity of these uncommon strains isolated. These results of this study may influence the redefinition of the vaccine strategy as the current vaccine used in Nigeria contains *P. multocida* B:3,4 and E:2. Furthermore more work needs to be carried out in other parts of the country in order to gather more relevant information with regards to capsular and somatic types.

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