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PIGMENTATION ANALYSIS REVEALS PYOCYANIN AS THE PREDOMINANT PIGMENT PRODUCED BY *Pseudomonas aeruginosa* COLONIZING WOUNDS/ULCERS

Ibrahim Y. Ngoshe[1], Tijani Isa[1], Muhammad M. Ibrahim[1*], Aisha Issa[2], Mustapha A. Isa[1], Liman M. Garba[3]

[1]Department of Microbiology, Faculty of Life Sciences, University of Maiduguri, Nigeria,

[2]Nigerian Institute of Medical Research, Maiduguri Station, Nigeria,

[3]Fidson Healthcare Plc, Nigeria

***Corresponding Author's Email Address**: mnistyle@yahoo.com

ABSTRACT

Pigment production is an important factor in the pathogenesis of Pseudomonas spp infection and could affect its prognosis. In addition to aiding a pathogen to evade the immune system and resist the antagonistic effect of antimicrobial drugs, pigments can induce tissue necrosis in infected wounds and disrupt the wound healing process. In this study, the infection of wounds/ulcers by Pseudomonas spp. and characterization of the type of pigment produced by isolates were analysed. Four hundred and twenty (420) wound swab samples were collected via the Levine technique, from patients attending various health centers in Maiduguri, Borno State, Nigeria. Samples were processed using standard microbiological procedure for the identification of Pseudomonas spp and suspect colonies were further confirmed using biochemical tests. Pigmentation was examined by the growth of Pseudomonas spp on Cetrimide agar. Seventy-two (72) samples yielded the growth of Pseudomonas spp, which gives a bacterial recovery rate of 17.1%. Three Pseudomonas spp were identified: P. aeruginosa (94.4%), P. putida (2.8%) and P. fluorescens (2.8%). Majority of Pseudomonas spp identified were Pyocyanin pigment producers (93.0%) followed by Pyoverdine (5.6%) and Pyomelanin (1.4%). Pigmentation across the three species revealed that 94.4% of P. aeruginosa were Pyocyanin producers and only one isolate produced Pyomelanin. All P. putida and P. fluorescens identified were Pyoverdine producers ($p<0.01$). Thus, analyzing pigment production in clinical isolates of Pseudomonas spp. can be an important tool that can provide an insight to their virulence status, and the disruption of pathways for the synthesis of these pigments can improve the prognosis of infections caused by Pseudomonas spp.

Keywords: Wound infection; *Pseudomonas* spp; Pigment; *P. aeruginosa*; Pyocyanin

INTRODUCTION

Pseudomonas spp. are diverse group of bacteria that are ubiquitous in nature. They are classified as gram-negative, motile, aerobic bacteria that have the versatility to survive in different niches. Some species such as *Pseudomonas aeruginosa* causes opportunistic infections in immunocompromised individuals [1,2]. Most members of the genus *Pseudomonas* produce a wide array of pigments that facilitate their adaptation in various habitats, including the human body. These pigments have been described as factors that increase the virulence of the bacterium by providing it with protection from host immunological response and the antagonistic effects of antimicrobial drugs [3,4]. Pigmentation is a complex characteristic in *Pseudomonas* spp. that can influence a variety of its invasive properties and increase its virulence and have been associated with the status or severity of infections, especially in infections caused by *P. aeruginosa* [5].

The most common pigment produced by most of *Pseudomonas* spp is Pyocyanin [6]. It is a blue-green pigment that plays a significant role in tissue destruction [7], the impairment of the host immune response [8], and the disruption of the normal functioning of host cells by inhibition of cellular respiration and energy production [9]. When these effects are combined with their ability to form biofilms [10] and resist antibiotics [11], *Pseudomonas* spp become a serious threat to human health. Other pigments produced by *Pseudomonas* spp include Pyoverdine (green) and Pyomelanin (brown). These two facilitate the expression of virulence factors and development of biofilms in *Pseudomonas* spp, which provides the ideal condition for the chronicity of infection [12,13] as well as the upregulation of iron uptake [12].

Pyoverdine can function in either iron acquisition or sequestration. It acts in the acquisition of ferric iron (Fe^{3+}) by binding it with high affinity leading to the extraction of iron sequestering proteins such as transferrin from the medium. This ensures the abundance of iron in the medium for utilization and also serves as an important factor in biofilm formation [14]. In an iron-limited environment, *P. aeruginosa* secretes pyoverdine which sequesters iron from the host leading to mitochondrial damage which leads to its dysfunction, especially in electron transfer and adenosine triphosphate (ATP) formation [15]. Pyoverdine can also act as a quorum-sensing molecule, which helps the bacterial coordination and enhance their ability to colonize a host [16].

Pigmentation produced by *Pseudomonas* spp. during infections can be a useful preliminary tool that can help in understanding its pathogenesis and additionally, it can be used in the phenotypic identification or serve as a component diagnostic tool for their identification [17]. This study tries to characterize the various species of *Pseudomonas* infecting wounds/ulcers of patients attending various hospitals in the northeastern city of Maiduguri. It also examines the production of one or more pigments by the various species of *Pseudomonas* identified.

MATERIALS AND METHODS

Study Area

The study was conducted in Maiduguri, the capital city of Borno State, Nigeria. Maiduguri shares borders with neighboring countries, such a Niger Republic, Chad, and Cameroon. Within Nigeria, Maiduguri shares borders with Adamawa, Yobe, and Gombe, and has Sahel savannah vegetation. The annual average temperature of Maiduguri ranges from 19.1°C to 34.7°C. It is a city with rich cultural heritage and home to the Kanem Borno Empire [18]. Four selected hospitals namely; University of Maiduguri Teaching Hospital (UMTH), State Specialist Hospital (SSH), Umaru

Shehu Ultra-Modern (USH), and Mohammed Shuwa Memorial Hospital (MSH) were included in this study.

Ethical Approval

Ethical approval was obtained from the research and Ethics Committees of the UMTH, SSH, MSH, USH and Borno State Hospitals Management Board (HMB) before commencement of the study. Informed consent of the patients was obtained before sample collection.

Inclusion criteria

This study included and was restricted to patients (in-patients and out-patients) that attended the selected hospitals (UMTH, SSH, MSH, and USH) and presented with wounds that belong to all four classes (Class I, II, III, and IV) of wound classification.

Exclusion criteria

Patients who attend other hospitals or clinics in Maiduguri, or patients that were attending the selected hospitals but presented with non-wound related cases were those excluded.

Sample Size Determination

The sample size (n) was calculated from the general formula as described by Daniel [19],

$$n= Z^2pq/d^2$$

Z= the standard normal deviate (1.96 which corresponds to 95% confidence level)

P= 51.5% (0.515) [20]

q= 1.0-p (1.0-0.515)

d= 0.05 (5%)

$$n = 383.$$

Taking care of possible attrition, additional thirty-seven (37) samples were added.

$$\text{Therefore, } n = 420.$$

Study Design, Sampling Technique and Sample Collection

This is a cross-sectional study where wound swab samples were collected (via simple-formal random sampling) from four hundred and twenty (420) patients attending the selected hospitals. Sample collection was done by adhering to standard aseptic technique to prevent contamination. The Levine technique was applied in the collection of wound swab samples and it involves rotating a swab over a 1 cm^2 region of the wound while applying pressure that will cause fluid to be released from the wound tissue [21]. Samples were collected from patients of both sexes (males and females) and various age groups. The swab containers were capped, labelled and taken to the Bacteriology Laboratory of the Department of Microbiology, University of Maiduguri, Nigeria for further analysis.

Bacteriological analysis (Phenotypic Identification of *Pseudomonas* spp)

The swab samples were directly inoculated on MacConkey and Blood agar and incubated for 18-24hours at 37^oC. Bacterial growth was phenotypically examined by the presence of visible bacterial

growth. Preliminary identification of *Pseudomonas* spp was based on colony characteristics of the organism which includes beta-hemolysis, non-lactose fermentation and pigment production on MacConkey and Blood Agar. Isolates were further sub-cultured onto freshly prepared plates of Cetrimide selective agar (Oxoid, UK) for the selective isolation of *Pseudomonas* spp. The plates were incubated overnight at 37^{o}C. The presence of *Pseudomonas* spp on the culture plates was determined qualitatively and quantitatively based on colony morphology or characteristics, and Gram stain reaction. Difference species were characterized and confirmed by biochemical tests.

Characterization of *Pseudomonas* spp using API NE

A single colony was picked up from a pure culture and made a suspension of it in sterile distilled water. The API_20NE Biochemical Test Strip which contains dehydrated bacterial media/bio-chemical reagents in 20 separate compartments was placed on the workbench. With the use of a Pasteur pipette the compartment of the API test strip was filled with the bacterial suspension, and these compartments (CIT, VP and GEL) were completely filled up. Sterile mineral oil was added into the ADH, LDC, ODC, H_2S and URE compartments. Drops of water were added in the tray before putting the API Test strip and the tray was closed. The tray was then marked with identification number and incubated at 37^{o}C for 18-24 hours. With the aid of an API Reading Scale (color chart) each test was marked as positive or negative on the lid of the tray. The wells were marked off into triplets by black triangles, for which scores were allocated. The scores were added up for only the positive wells in each triplet and then the supplementary test oxidase was included in the profile. The data was then analysed by the manufacturer's software and positive results with ≥89% probabilities were confirmed as *Pseudomonas* spp.

Pigmentation analysis

Pigment production was evaluated based on Pseudomonal growth on Cetrimide agar medium; targeted pigments were Pyocyanin (Blue Green, Greenish) Pyoverdine (Yellow, yellowish Green) Pyomelanin (Red or Brown). Pigment production was further confirmed by bacterial inoculation on API_20NE Biochemical Test Strip.

Data Analysis

Data were analyzed as frequencies and percentages and presented in tabular form. Statistical analysis was conducted using Chi square test at 95% level of significance (α=0.05) [22].

RESULTS AND DISCUSSION

Analysis of Culture Positivity, Specie type and Pigmentation by *Pseudomonas* spp Identified from the Wounds of Patients Attending Hospitals in Maiduguri.

Isolation and identification of *Pseudomonas* spp to specie level was conducted (Table 1). Seventy-two (72) out of four hundred and twenty (420) swab samples yielded positive culture for *Pseudomonas* spp. This corresponds to bacterial recovery rate of 17.1%. Three *Pseudomonas* spp were identified, of which *P. aeruginosa* (94.4%) was predominant, followed by *P. putida* (2.8%) and *P. fluorescens* (2.8%). A significant majority of *Pseudomonas* spp identified were Pyocyanin pigment producers (93.0%) followed by Pyoverdine pigment producers (5.6%) and Pyomelanin producers (1.4%).

Table 1: Analysis of pigmentation and distribution of *pseudomonas* spp identified from the wounds of patients attending hospitals in Maiduguri

Swab Samples analyzed for *Pseudomonas* spp	**Frequency**	**Percentage freq. (%)**	**Cumulative freq. (%)**
Positive	72	17.1	17.1
Negative	348	82.9	100
Total	420	100	
Pseudomonas spp			
Pseudomonas aeruginosa	68	94.4	94.4
Pseudomonas putida	2	2.8	97.2
Pseudomonas fluorescens	2	2.8	100
Total	72	100	
Pigmentation			
Pyocyanin (Blue Green, Greenish)	67	93.0	93.0
Pyoverdine (Yellow, yellowish Green)	4	5.6	98.6
Pyomelanin (Red or Brown)	1	1.4	100
Total	72	100	

Characterization of *Pseudomonas* spp and Pigment Production

The analytical profile index for the characterization of *Pseudomonas* spp showed an average API sensitivity of 98.0%. Isolates have shown intra-specie differential pigmentation. 55.6% of *P. aeruginosa* isolates showed blue-green pigmentation (Pyocyanin), 37.5% produced green pigment (Pyocyanin) while 1.3% produced brown pigment (Pyomelanin). The two isolates each belonging to *P. putida* and *P. fluorescens* specie produced yellow (2.8%) and yellowish-green (2.8%) pigmentation respectively. As such they can be classified under Pyoverdine pigmentation. Analysis of inter-specie pigmentation was statistically significant ($p<0.01$) (Table 2; Table 3).

Table 2: Analytical profile index (API) for the characterization of *Pseudomonas* spp found in wounds/ulcers

Analytical profile Index isolates	Identification (%)	No of strains	Numerical codes	Pigmentation (Colour)	Total no of spp
Pseudomonas aeruginosa	98.8	40	2202046	Pyocyanin (Blue-green)	
Pseudomonas aeruginosa	98	27	2216004	Pyocyanin (Green)	68 (94.4)
Pseudomonas aeruginosa	97	1	2216000	Pyomelanin (Brown)	

Pseudomonas putida	98	2	2203004	Pyoverdine (Yellow)	2 (2.8)
Pseudomonas fluorescens	98	2	2203004	Pyoverdine (Yellowish green)	2 (2.8)
Total	-	72	-	-	72 (100)

Table 3: Distribution of various pigments produced by *Pseudomonas* spp identified from wounds/.ulcers of patients attending selected hospitals in Maiduguri

Pseudomonas spp	Pigmentation (%)					*p* value	Total
	Pyocyanin		Pyomelanin	Pyoverdine			
	Blue-green	Green	Brown	Yellow	Yellowish		
Pseudomonas aeruginosa	40 (55.6)	27 (37.5)	1 (1.3)	0 (0.0)	0 (0.0)		68 (94.4)
Pseudomonas putida	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.8)	0 (0.0)	0.001	2 (2.8)
Pseudomonas fluorescens	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.8)		2 (2.8)
Total	40 (55.6)	27 (37.5)	1 (1.3)	2 (2.8)	2 (2.8)	-	72 (100)

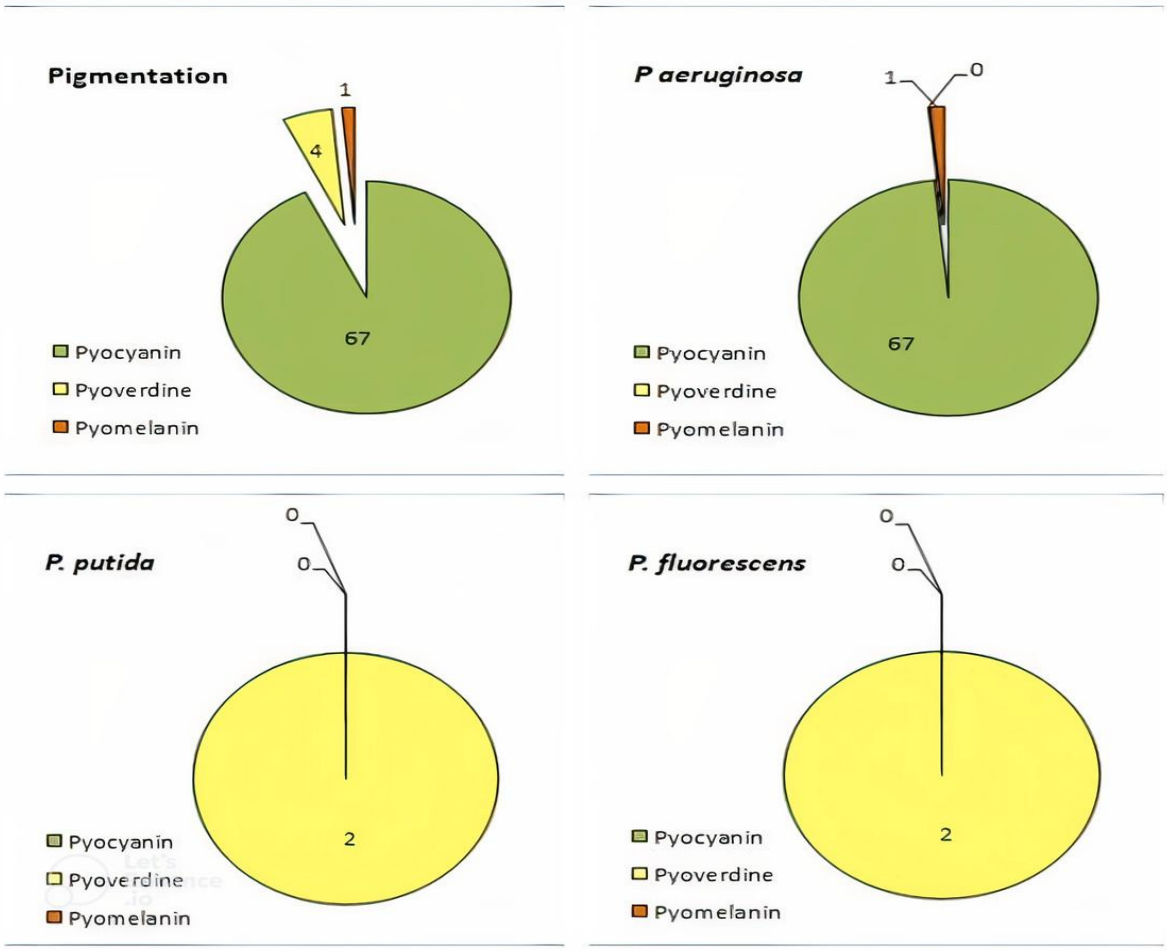

Figure 1: Comparative analysis of pigments produced by *Pseudomonas* spp identified from wounds/ulcers of patients attending various hospitals in Maiduguri

In this study of wound infection by *Pseudomonas* spp, we observed that three distinct species comprising of *P. aeruginosa*, *P. putida* and *P. fluorescens*, were colonizing the wounds of patients examined. It is well established that these three species can cause infection mostly in immunocompromised individuals with varying degrees of virulence and pathogenicity. Similar studies on wound infection elsewhere have reported the identification of *P. aeruginosa* [23], *P. putida* [24], and *P. fluorescens* [25]. As is the main characteristic of the sample population for this study, exposed skin abrasions or wounds can serve as an ideal niche for colonization by *Pseudomonas* spp which can lead to serious life threatening infection. We identified *P. aeruginosa* as the most predominant *Pseudomonas* species colonizing wounds and was found in more than 94.0% of infected wounds. *P. aeruginosa* is a highly virulent and most common opportunistic pathogen that is encountered, especially in the nosocomial setting [26]. Apart from secreting toxins and substances that impair the wound healing process [27], *P. aeruginosa* is implicated in both localized (in-situ) and systemic infections such as bacteremia [28]. Such infections are of utmost concern due to high mortality, especially in immunocompromised patients [29]. Its presence in a wound can lead to the aforementioned diseases, especially if left unattended for a long period of time.

This study revealed that *P. fluorescens* and *P. putida* were infecting wounds albeit in very low proportions. *P. fluorescens* are found in low abundance in various human anatomical sites such as lungs, mouth and abdomen where it functions as a normal flora [30,31,32]. It is far less virulent than *P. aeruginosa*. However, it can cause acute infections in humans and mostly gains entry into the bloodstream where it causes bacteremia. Most cases of bacteremia have been attributed to the transfusion of poorly screened blood or contaminated blood products [33]. Other areas where *P. fluorescens* are found include bone [34], cerebrospinal fluid [35], uterus [36] and the urinary tract [37]. Most infections caused by *P. putida*, on the other hand, are noscomially acquired and the risk factor can be attributed to the use of medical devices [24]. *P. putida* is implicated in the aetiology of several other diseases such as skin and soft tissue infections (SSTIs) [38,39], pneumonia, tonsillitis and thrombophlebitis [40], cholangitis, cholecystitis and catheter-related blood stream infections [41].

A significant majority of *P aeruginosa* (99.0%) identified in this study were Pyocyanin pigment producers. Only one isolate produced pyomelanin. Pyocyanin plays a significant role in tissue destruction [7] and as such, can enhance further wound damage and retard the wound healing process. In the event that a Pyocyanin producing *P. aeruginosa* gains access from the wound into systemic circulation, it can cause a persistent and invasive infection due to the pigment's role in circumventing the host immune response [8], in addition to disruption of cellular function [9]. Both *P putida* and *P. fluorescens* identified in this study were Pyoverdine pigment producers and that should not be underestimated because Pyoverdine facilitates the development of biofilms which is a recipe for chronicity of infection [12].

Biofilm production is a means of shielding a pathogen residing in a wound from the antagonistic action of antimicrobial drugs and clearance by the immune system. Pyoverdine can also modulate the interaction and signaling between *Pseudomonas* spp through the process of quorum sensing [16]. This is important for the establishment of infection and communication especially in a population of bacteria living in a biofilm matrix. This provides an ideal ecosystem for the exchange of virulence genes between different strains of the same species in a single –specie biofilm. The resultant scenario is the restriction of treatment options and poor prognosis of the wound/ulcer.

CONCLUSION

We report the isolation of three (3) *Pseudomonas* spp, which comprise of *P. aeruginosa*, *P. putida* and *P. fluorecens*, from patients' wounds/ulcers. We also revealed that *P. aeruginosa* is the most abundant of the three species identified. Analysis of pigmentation revealed that *P. aeruginosa* were predominantly Pyocyanin pigment producers, and only one isolate produced Pyomelanin while *P. putida* and *P. fluorecens* produced Pyoverdine pigment respectively.

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CONFLICT OF INTEREST

The authors hereby declare that no conflict of interest exists.

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