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Molecular identification and virulence factors of pseudomonas aeruginosa strains isolated from animal products

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen which is characterized by its capacity to develop resistance and virulence factors. Despite their knowledge, these resistance and virulence factors are not more characterized at level of strains from local food. This study aimed to detect the presence of various virulence factors and metallo-β-lactamase-producing P. aeruginosa from animal products. One hundred (100) strains of Pseudomonas aeruginosa were isolated from bovine meat (60), fresh fish (24) and smoked fish (16). API20NE methods, IPM-EDTA disk method and polymerase chain reaction (PCR) using the rpoB, lasB, exoS, algD, and plcH gene were performed. The rpoB gene confirmed 99.0% of presumptive strains as *Pseudomonas aeruginosa*. All of virulence genes studied were detected in decreasing importance order, lasB (89.0%), exoS (84.0%), algD (73.0%) and plcH (71.0%) ($p<0.05$). A percentage of 22.0% of animal strains were P. aeruginosa producing MBLs. The simultaneous determination of virulence factors and metallo-β-lactamase is of interest for the efficacy of surveillance of infections associated with P. aeruginosa.

Keywords: *pseudomonas aeruginosa*, mbls, bovine meat, fresh fish, smoked fish, PCR

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Introduction

Microorganisms are the primary cause of food spoilage and food borne illness.[1,2] Among these germs, *Pseudomonas aeruginosa* occupies a large place in food contamination. It contaminates domestic and wild animals, human beings, plants, drinking water, and is also isolated from variety foods.[3,4] This psychrotrophic germ is specific spoilage organisms (SSO) of meat, poultry and fish.[4]

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting virtually all tissues.[5] It possesses a variety of virulence factors that may contribute to its pathogenicity.[5,6] It has also a large number of virulence factors such as, Exo enzyme S, encoded by the exoS gene that is secreted by a type-III secretion system.[5,6] Phospholipases C (PLCs) are also implicated in virulence. *Pseudomonas aeruginosa* produces three PLCs: one with hemolytic activity (PlcH), one other non-hemolytic (PlcN) and the last, a PLcB which is important for chemotaxis and plays an important role in "twiching" mobility.[5–7]

A phosphate deficiency induces the production of PlcH. These PLCs are secreted via the type-II secretion system.[5,6] Others genes are implicated in *Pseudomonas aeruginosa* virulence: LasB elastase, a zinc metalloprotease encoded by the LasB gene, has an elastolytic activity on lung tissue.[5,7] Mucoid colonies composed of alginates produced by *Pseudomonas aeruginosa*, involving algD genes protect the bacteria from the host immune responses and biocides (antiseptic, disinfectant and antibiotic).[6,8]

This production of alginates therefore makes the strains of *P. aeruginosa* multi-resistant. These multi-resistant strains can harbor and produce certain resistance genes such as metallo-β-lactamase (MβL).[9,10] Those metallo-enzymes are capable to inactivate β-lactam antibiotics such as carbapenems, penicillins and cephalosporins.[10–12] These enzymes promote the involvement of *P. aeruginosa* in foodborne diseases and nosocomial infections in various regions of the world.[2,10,13] The strains of *P. aeruginosa* metallo-β-lactamase-producing (MβL) are also responsible for serious infections such as septicemia and pneumonia.[14] Thus, several phenotypic methods can be used to determine strains of *Pseudomonas aeruginosa* producing metallo-β-lactamase of various origins.[9,15]

In addition, PCR allows rapid and complete identification of *Pseudomonas aeruginosa*.[5,16] This method also allows the determination of resistance and virulence genes associated with this bacterium. The relationship between animal products, resistance to antimicrobials and virulence factors of *Pseudomonas aeruginosa* are not widely studied. This study aimed to detect the presence of various virulence factors and metallo-β lactamase-producing *P. aeruginosa*.

Materials and methods

Isolation of P. aeruginosa from animal products

The studies were carried out in and around market of Abidjan district (Côte d'Ivoire). A total of one hundred (100) isolates of *P. aeruginosa* were used in the present study. The isolates were obtained from different samples of animal products, including beef (57), fresh fish (23) and smoked fish (18). The samples were collected from local market and put in sterile Stomacher bags, stored at 4°C, and analyzed within 30minutes of collection. Ten (10) g of the flesh of each bovine meat, fresh fish and smoked fish sample were homogenized in 90ml peptone water, and then, serial decimal dilutions were prepared.

Amount of 0.1ml of each dilution was spread on the selective medium Pseudomonas cetrimide agar (PCA) using a spreading technique. Plates were incubated at 44°C for 18-24hours and observed for suspected colonies of *P. aeruginosa*.

Morphological and biochemical characterization

Gram's reaction- Gram's staining, Motility determination-Hanging drop method and Motility test were carried out for the morphology of cell. Biochemical identification of the isolates was carried out using API 20NE (bioMérieux, Marcy l'Etoile, France) and the API database.

Phenotypic detection of MBL activity

All strains that showed reduced susceptibility to imipenem ≥8g/ml were screened for MBL production. These strains were subjected to a phenotypic analysis by EDTA (Sigma Chemicals, St. Louis, MO) combination disk test.[17] Briefly, a 18hours culture of animal isolate was diluted with peptone water (Oxoid, USA) corresponded to the 0.5 McFarland standard, which is approximately 108CFU/ml and spread on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, England) plate using cotton swab. Two IPM (10µg) disks were placed on the surface of the agar at distances of 20mm away from each other. Then, 4µL of EDTA (0.5 M, pH 8) solution was added to one of the IPM (10µg) disks. Another IPM disk (10µg) was placed at 20mm center to center of a sterile non-impregnated disk on which 10µl of EDTA (0.5 M, pH 8) has been added.

The inhibition zones displayed around the IPM and the IPM-EDTA disks were compared after 18 to 24hrs incubation at 37°C. The difference of ≥7mm between the inhibition zone diameter of the IPM-EDTA disk and that of the IPM (10µg) alone disk was considered to be a positive test for the presence of MBLs.[14] *P. aeruginosa* PA105663 producing IMP-7; *P. aeruginosa* PS679/00 producing VIM-2; and *P. aeruginosa* ATCC 27853 were used as positive and negative controls.

Molecular Identification of Pseudomonas aeruginosa

Extraction and purification of DNA: Template DNA was extracted from whole organisms by boiling [18]. Bacteria were harvested from an overnight broth culture (Biokar Diagnostics, BK015HA, France), suspended in 1ml sterile Milli-Q water (milli-Q™, Millipore Corporation, USA). A suspension of 200µl was incubated at -20°C for 15minutes and boiled at 95°C for 15minutes.

The suspension was immediately cooled at 4°C for 10minutes and then centrifuged at 14 000 rpm for 10minutes to pellet the cell debris. The DNA template was purified according to the method described by Zimmermann et al.[19] The purity and DNA concentration of the extract were determined by spectrophotometer (Eppendorf BioPhotometer plus, USA).

PCR amplification

Single PCR for characterization of rpoB gene was carried out with a total volume of 25µl consisted of 16µl of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5µl of 5XTP, 1.5µl of MgCl2 (2mM), 0.2µl of dNTPs (10mm), 0.1µl of each primer (20mM) (Integral DNA Technology, California, U. S. A) (rpoB F: Order No. 2512433, Ref. No. 70393602; rpoB R: Order No. 2512433, Ref. No. 70393603), 0.1µl of Go tag polymerase (Promega Corporation, Madison, WI 53711-5399, USA) and 2µl of DNA matrix. DNA rpoB region amplification was performed using the primer set rpoB F (5'-CAGTTCATGGACCAGAACAACCCG-3') and rpoB R (5'ACGCTGGTTGATGCAGGTGTTC-3'), aligning on positions 1552 and 2298 of the rpoB gene sequence of *Pseudomonas aeruginosa* UCBPP-PA14 (CP000438).[20–22]

The rpoB DNA was amplified using the following protocol: initial denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min and extension at 72°C for 2min, with a single final extension of 7min at 72°C. Amplification was performed with 10µl of PCR products which were separated in 1.5% agarose gel for 30min at 120V. After amplification, the agarose gel was put in ethidium bromide (0.5µg/ml) and detected by Molecular Imager Gel DocTM EZ (Bio-Rad, USA).

Multiplex PCRs were used to detect lasB, exoS, algD and plcH. PCR mixtures with a final volume of 25µl consisted of 15.8µl sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5µl 5XTP, 1.5µl MgCl2 (2mM), 0.2µl dNTPs (10mM), 0.1µl each primer (20mM) (Integral DNA Technology, France), 0.1µl Go tag polymerase (Promega Corporation, Madison, WI 53711-5399, USA) and 2µl DNA template. A quantity of 2µl of sterile Milli-Q water was used for negative control and DNA of ATCC reference strain 27853 used for positive control.

The oligonucleotide primers used in this study and the amplification program are listed in Table 1. Each PCR was performed using thermocycler type T3000 thermocycler, Block type standard 3a, (Biometra, Germany). The amplified DNA was separated by gel electrophoresis with 2% agarose containing 0.5µg/ml with ethidium bromide for 30min at 130V, visualized under UV transillumination and photographed (Molecular Imager Gel DocTM XR+, Bio-Rad). Amplified genes were identified on the basis of fragment size shown in Table 1.

Table 1 Primers used for amplification of virulence genes in multiplex PCR

Primers	Target gene	Sequence (5'-3')	Product size (bp)	Amplification program	Annealing temperature (°C)	Source
LasB-F LasB-R	LasB	GGA ATG AAC GAG GCG TTC TC GGT CCA GTA GTA GCG GTT GG	300	94°C, 5min 35 x [94°C, 35s ; 60°C, 1min ; 72°C,1min] 72°C, 7min ; 4°C …	60	[23]
ExoS-F ExoS-R	exoS	CTT GAA GGG ACT CGA CAA GG TTC AGG TCC GCG TAG TGA AT	504			[23]

Table Continued

algD-F algD-R	algD	ATG CGA ATC AGC ATC TTT GGT CTA CCA GCA GAT GCC CTC GGC	1310	94°C, 5min 35 x [94°C, 35s ; 61°C, 1min; 72°C, 1min]	62	[23]
plcH-F plcH-R	plcH	GAA GCC ATG GGC TAC TTC AA AGA GTG ACG AGG AGC GGTAG	307	72°C, 7 min; 4°C …	60	[23]

Statistical analysis

The statistical analysis was carried out on the software Statistical Package for the Social Sciences (SPSS) 20.0 (IBM SPSS, Chicago, IL, United States of America) using the Student's t test, Mann-Whitney U test, Spearman's correlation analysis and multiple regression analysis. Statistical significance was set at $p<0.05$.

Results

Morphological and biochemical characterization

Pseudomonas aeruginosa is a motile, Gram negative, facultative, rod-shaped bacterium measuring 0.5 to 0.8µm by 1.5 to 3.0µm in size. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. Its optimum temperature for growth is 37°C, but retains a growth potential at temperatures as high as 42°C. *P. aeruginosa* strains produce two types of soluble pigments, a fluorescent pigment pyoverdin, and a blue pigment, pyocyanin.

Pseudomonas aeruginosa strains isolated

Out of 112 presumptive isolates of *Pseudomonas aeruginosa*, 101 (90.1%) were identified by API 20NE (Table 2). The rpoB gene confirmed 100 (99.0%) of these strains as *Pseudomonas aeruginosa* out of 101 (90.1%) (Figure 1).

Prevalence of virulence genes among all studied P. aeruginosa isolates

Figure 2 to Figure 4 showed that all the virulence genes studied in this study were detected in the 100 strains of *Pseudomonas aeruginosa*. According to the Figure 2 and Figure 3 lasB gene with 89.0% has been most detected following by exoS (84.0%). The prevalence of algD and plcH gene was respectively 73.0% and 71.0% (Figure 2) (Figure 4).

Prevalence of Pseudomonas aeruginosa virulence genes in animal products

The prevalence of virulence genes studied was higher than 50% in all animal products analyzed. The prevalence of lasB and exoS genes was respectively higher in bovine meat (96.7%, 93.3%), followed by smoked fish (87.5%, 75.0%) and fresh fish (70.8%, 66.7%) (Figure 5). That of algD and plcH gene was respectively higher in fresh fish (79.2%, 75.0%) followed by bovine meat (75.0%, 71.1%) and smoked fish (56.3%, 62.5%) (Figure 5).

Metallo-ß actamase and virulence patterns

The strains of *Pseudomonas aeruginosa* producing elastase (lasB) and harboring the alginate (algD) showed the same percentage of blaIMP genes 73.3% (Figure 6). Those strains whish secreted exoenzyme (exoS) and harboring the alginate (algD) showed the same percentage of blaVIM genes 66.7% (Figure 7). The strains of *Pseudomonas aeruginosa* harboring the PlcH gene indicated a production of metallo-β lactamase (VIM and IMP) of less than 50% (Figure 6).

Screening for and confirmation of MBLs

Phenotypic methods indicated that 22 (22.0%) of the 100 strains studied were *P. aeruginosa* producing MBLs using (Figure 7).

Table 2 Frequency of strains confirmed by the API 20NE and rpoB genes

Number of isolates presumptive *p. aeruginosa* N=112		
Identification of P. aeruginosa	Phenotypic and biochemical (API 20NE)	Molecular (rpoB gene)
Effective (N)	101	100
Percentage (%)	90.1	99

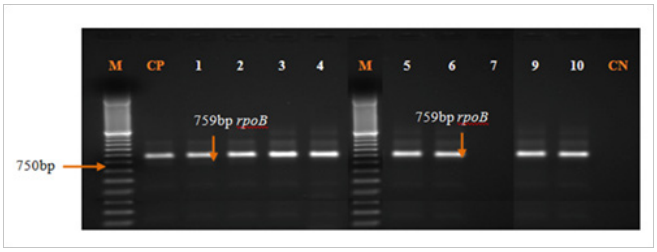

Figure 1 rpoB profiles of *Pseudomonas aeruginosa* isolates. Lanes 1-4; 5-6; 9-10: *Presence of Pseudomonas aeruginosa* inanalyzed products; Lane 7: Absence of *Pseudomonas* in analyzed products; CP: Positive control (*Pseudomonas aeruginosa* ATCC 27853); CN**:** Negative control; M: Marker Gene Ruler 100bp (Bench Top, 100bp DNA Ladder, Promega Corporation, USA).

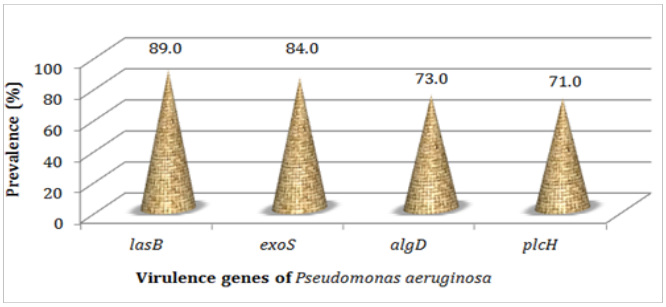

Figure 2 Prevalence of virulence genes among all studied P. aeruginosa isolates. LasB, elastase LasB-encoding gene; exoS, exoenzyme S-encoding gene; algD, GDP-mannose 6 dehydrogenase AlgD (alginate)-encoding gene; plcH, haemolytic phospholipase C precursor-encoding gene.

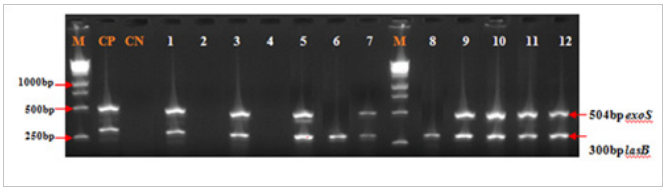

Figure 3 Electrophoretic profile of amplification products of virulence genes exoS and lasB in analyzed products. Virulence genes were present in analyzed products, Lane 1,3,5,7,9,10,11 and 12: exoS and lasB; Lane 6 and 8: lasB; Virulence genes were absent in analyzed products, Lane 2 and 4. M: Marker Gene Ruler 250bp (Bench Top, 1kb DNA Ladder, Promega Corporation, USA); lasB, elastase LasB-encoding gene; exoS, exoenzyme S-encoding gene.

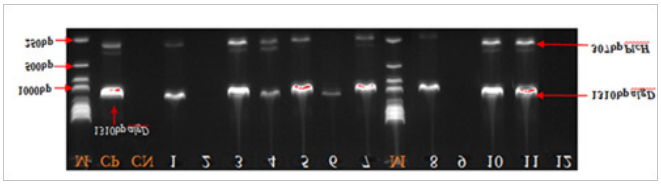

Figure 4 Electrophoretic profile of amplification products of virulence genes algD and PlcH in analyzed products. Virulence genes were present in analyzed products, Lane 1,4,5,7,8,10 and 11: algD and PlcH; Lane 3 and 6: algD; Virulence genes were absent in analyzed products, Lane 2, 9 and 12. M: Marker Gene Ruler 250bp (Bench Top, 1kb DNA Ladder, Promega Corporation, USA); algD, GDP-mannose 6-dehydrogenase AlgD (alginate)-encoding gene; plcH, haemolytic phospholipase C precursor-encoding gene.

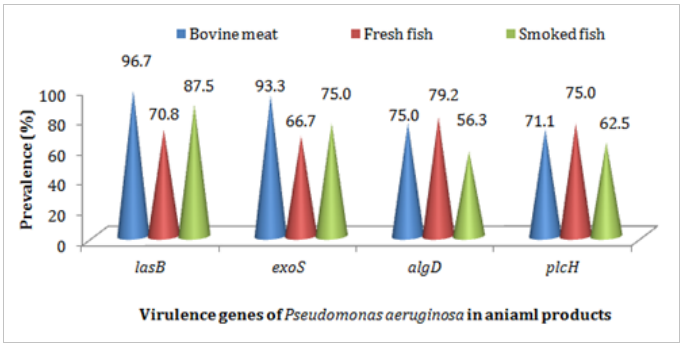

Figure 5 Prevalence of Pseudomonas aeruginosa virulence genes in animal products.

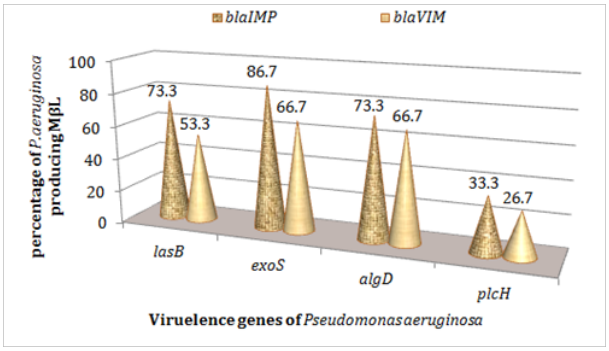

Figure 6 Percentage of metallo-β lactamase according to virulence genes of Pseudomonas aeruginosa. MβL Imipenemase (IMP), Verona imipenemase (VIM).

Figure 7 Metallo-β lactamase (MBLs) producing by Pseudomonas aeruginosa isolated from animal products.
The test showed difference of ≥7mm between the inhibition zone diameter of the IPM-EDTA disk and that of the IPM (10µg) alone disk. The test was considered to be positive for producing of MBLs. Imipenem (IPM); 1: IMP disks (10µg)+4µlEDTA (0.5M, pH 8); 2: IMP disks (10µg) without EDTA (0.5M, pH 8); 3: non-impregnated disk+10µlEDTA (0.5M, pH 8); 4: IMP disks (10µg) without EDTA (0.5M, pH 8).

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Discussion

Morphological and biochemical characteristics confirmed that *Pseudomonas aeruginosa* is a motile (unipolar), gram negative, facultative, catalase positive, oxidative positive, nitrate reduction positive, non-fermentative and rod-shaped bacterium measuring 0.5 to 0.8µm by 1.5 to 3.0µm in size. Its optimum temperature for growth is 3°C, but retains a growth potential at temperatures as high as 42°C. *P. aeruginosa* strains produce two types of soluble pigments, a fluorescent pigment pyoverdin, and a blue pigment, pyocyanin. These results are consistent with those found by Virupakshaiah & Hemalata[2] who identified *Pseudomonas aeruginosa* from food isolates.

Identification of *P. aeruginosa* has traditionally relied on phenotypic methods. This still is the most accurate standard when dealing with typical isolates of *P. aeruginosa*. Moreover, biochemical testing takes long time to perform and requires extensive hands-on work by the technologist. Molecular methods have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa*.[5,16] Molecular identification by using rpoB gene showed 100 (99.0%) of the strains were *Pseudomonas aeruginosa*. This result also indicated the strong discriminating power of the identification method using rpoB gene and confirmed the heterogeneity of the *Pseudomonas aeruginosa* observed by some authors.[5,16] This high molecular identification rate showed that genomic studies are needed to confirm the exact taxonomic position of *Pseudomonas aeruginosa*.

The results showed also that all the virulence genes studied in this study were detected in the 100 strains of *Pseudomonas aeruginosa*. Therefore, strains isolated from bovine meat, fresh fish and smoked fish could host these virulence genes studied. Analysis revealed lasB gene (89.0%) and exoS (84.0%) were the most detected in this study and this could be justified by the fact that *P. aeruginosa* secretes elastase (LasB); a metalloproteinase involved in the host colonization and tissue damage.[5] Previous studies have shown a high prevalence of the lasB gene in P. aeruginosa, irrespective of their isolation origin.[5,6,24] The prevalence of ExoS observed in this study shows that the strain of *Pseudomonas aeruginosa* of animal origin could be involved in tissue destruction; ExoS gene is involved in lung infection and may be important for bacterial dissemination.[6,7,24]

The high prevalence of algD (73.0%) gene observed in this study could be at the origin of the conversion of *Pseudomonas aeruginosa* strains to a mucoid phenotype overproducing alginates.[6,25] This alginate prevalence indicates that isolated strains of animal products are involved in the formation of biofilms as alginates have been widely regarded as the major exopolysaccharides of the biofilm matrix.[24,25] The prevalences of plcH (71.0%) gene showed that strains isolated were able of secreting hemolytic exoenzyme and phospholipase C and thus strains could be involved in pulmonary infections.[26,27] Our results obtained in this study correlate with those proved by Bradbury et al.,[28] who proved that *P. aeruginosa* isolated from nosocomial infections were found to have an increased prevalence of virulence genes.

This study also showed that *P. aeruginosa* producing elastase (lasB) and harboring the alginate (algD) showed the same percentage of blaIMP genes (73.3%). Those strains whish secreted exoenzyme (exoS) and harboring the alginate (algD) showed the same percentage of blaVIM genes 66.7%. The strains of *Pseudomonas aeruginosa* harboring the PlcH gene indicated a production of metallo-β lactamase (VIM and IMP) of less than 50%. The prevalence of VIM and IMP was relatively high in the various animal products and therefore animal strains could be involved in serious infections.[9,13,29] These same strains could also be involved in zonoses. The detection of virulence factors and metallo-β lactamase in *P. aeruginosa* can prevent gene mutations and reduce the risk of multi resistance linked to this bacterium.

Conclusion

The simultaneous using of biochemical methods and rpoB genes provides more confident detection of *P. aeruginosa* by PCR. Determination of different virulence and resistance genes of *P. aeruginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. Study indicated that MBL-producing isolates were associated with virulence factors and responsible for serious infections. Thus, good practices in hygiene procedures and food preparation are necessary to control and reduce biological risks.

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None.

Conflict of interest

The author declares no conflict of interest.

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