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Phenotypic and Genotypic Characterization of Extended Spectrum *β*-Lactamase *Klebsiella pneumoniae* and Fluorescent *Pseudomonas* spp. Strains from Market Garden Products and Their Watering Water in Benin (West Africa)

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Abstract

Market garden products can carry several types of microorganisms, and their consumption is the source of many cases of food poisoning. This work aimed to improve food safety in Benin. In characterizing strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. at the biochemical and molecular level, the target was to identify contaminated watering water and garden products sold during Cotonou in both the dry and rainy seasons. A total of 164 samples of market garden products and 22 samples of watering water were investigated. The results showed that 5.91% of market garden products and watering water were contaminated by *K. pneumoniae* and 20.43% by fluorescent *Pseudomonas* spp. During the dry season, cabbage was most contaminated by fluorescent *Pseudomonas* spp. (50%). Pool water was more contaminated with *K. pneumoniae* (17%). All isolated strains were resistant to both amoxicillin and penicillin. All strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. were not resistant to imipenem, and 22% of them produced penicillinase. Among the 49 strains producing penicillinase isolated, 64.29% and 21.43% carried bla_{TEM} and bla_{SHV} respectively while 14.28% carried bla_{CTX-M} genes. In light of the previously-developed results and considering the importance of horticultural products in Beninese food habits, we must improve national awareness of the risk for foodborne illness.

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1. Introduction

In West Africa, commodity garden products or "commodity food" is eaten almost daily by urban and rural households rather than "luxury goods" essentially consumed in urban areas, restaurants and multi-person households [1]. In Benin, the production of vegetables is an important source of jobs in urban and surrounding areas, especially the riverside or valleys of some areas [2]. In southern Benin, the incomes generated by vegetable production allow ten thousands of families to satisfy their needs [3]. Urban agriculture has become important in Africa today because of its spatial visibility, with many small gardens for the pleasure of visitors [4]. Due to these factors, vegetables are essential for the health and preservation of the human species. Under such conditions, increasing the consumption of vegetables is therefore the best way to improve the quality of the diet. Thus, over the past two decades, agriculture has been much developed in urban and surrounding areas in West Africa with rapid urbanization and a strong economic concentration [5]. The lack of financial means for the supply of water and synthesis of soil fertilizers is the reason why vegetable farmers use wastewater from swamps and other sources for irrigation and manure from animals as fertilizer for the soil [6] [7]. These practices promote serious contamination of vegetables by microorganisms that might prove dangerous to the consumer. The factor usually involved in the contamination of vegetables is the watering water [7] [8]. When livestock manure is near the vegetables, it can contaminate the water with millions of *Enterobacteriaceae*. In addition, flowing storm water also carries microorganisms coming largely from domestic or wild animal defecation [9].

The microorganisms responsible for the contamination of watering water and market garden products are generally *Enterobacteriaceae*.

Indeed, *K. pneumoniae* and *Pseudomonas* spp. are components of the commensal flora of the mucous membranes and upper respiratory tract. They are widely found in the environment (waste water, soils, plants, etc.). Strains of *K. pneumoniae* are some important pathogens, and they are often the cause of nosocomial pneumonia (7% to 14% of cases), septicaemia (4% to 15%), urinary infections (6% - 17%), infections of wounds (2% - 4%), infections in the intensive care unit (4% to 17%) and neonatal septicemia (3% to 20%) [10]. They are also major opportunistic pathogens, especially in immune-suppressed individuals. Meanwhile, *Pseudomonas* spp. are opportunistic pathogens that often invade host tissues and cause infection and bacteremia in immune-suppressed hosts (e.g., those with HIV/AIDS, cystic fibrosis of the pancreas, bronchiectasis and severe chronic obstructive pulmonary disease, burns, malignant disease or diabetes mellitus, etc.) [11].

However, there are some antibiotics that destroy these *Enterobacteriaceae*. Only E*nterobacteriaceae* microorganisms have generally developed various strategies to change the potential for action of these molecules [12]. Resistance to extended-spectrum cephalosporins in the family of *Enterobacteriaceae* has commonly been associated with the expression of extended-spectrum TEM and SHV *β*-lactamases (ESBLs). Ten (10) variants of the CTX-M-type *β*-lactamases have been described in various enterobacterial species [13].

It therefore appears necessary to evaluate the risks of microbial pollution from watering water in vegetable products and horticultural products and consider the importance and ever-growing place of these products in the diet of the population of Benin. This study was undertaken in this light and is designed to characterize the strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. producing *β*-lactamase inhibitors and identify contaminated market garden products and watering water. The results show that most of these products are consumed directly at Cotonou in Benin; this study was also conducted to assess and to limit the health risks associated with consumption of these products and restrict non-antibiotic prescription.

2. Material and Methods

2.1. Sample Collection of Market Garden Products

Four market garden products, including lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), great nightshade (*Solanum macrocarpum*) and carrot (*Daucus carota*), were collected from 4 truck farming sites (**Figure 1**) (Fid jrossè-Jacquot, Barrier-Asecna, Akpakpa Rigle-Range and ONIP Cadjèhoun) in Cotonou (Benin). These sites
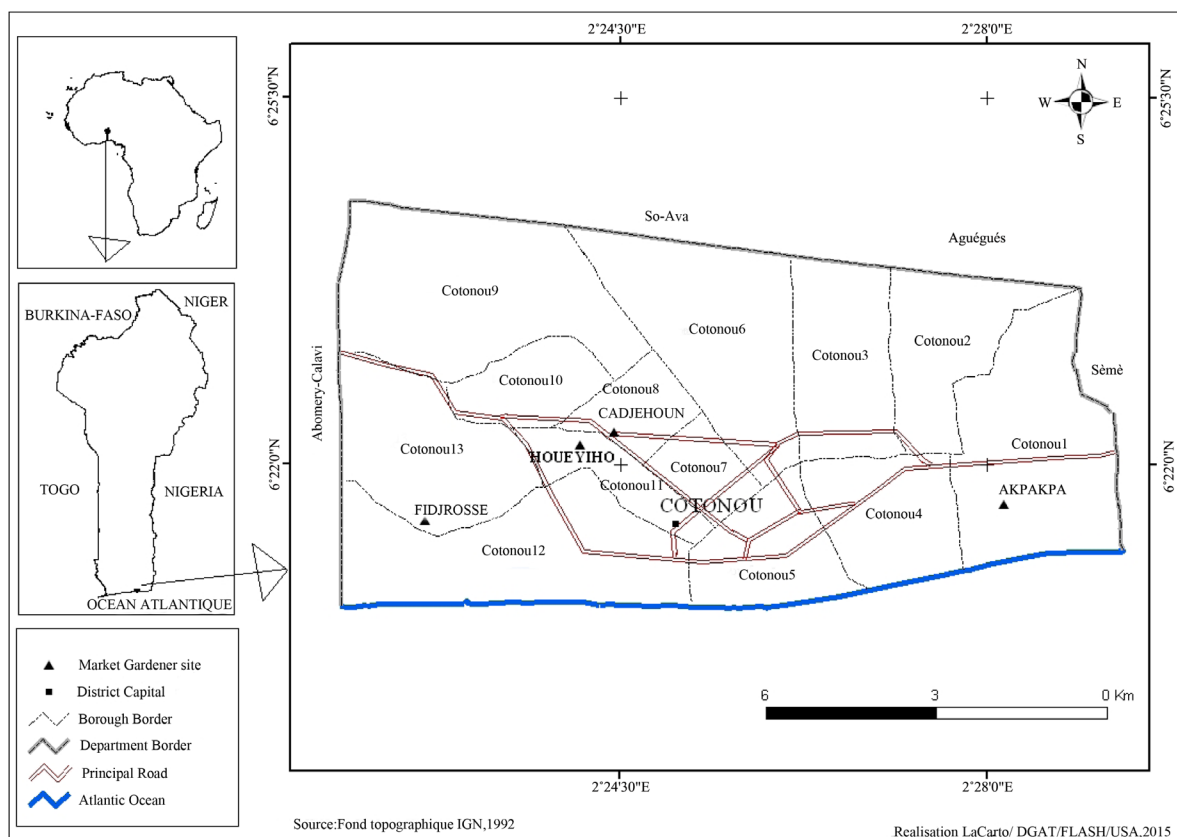
Figure 1. Overview of the garden sites.

were selected after an initial investigation. At each site, three gardeners were randomly selected among those who grew the four targeted products, and two samples of each product were collected per gardener. The samples were collected both in the dry season (January to February 2013) and in the rainy season (October to November 2013). During the dry season, 82 samples (22 lettuces, 22 cabbages, 22 great nightshades and 16 carrots) were collected, because the carrot samples that were to be collected at ONIP Cadjèhoun were not found. During the rainy season, 82 samples (22 lettuces, 16 cabbages, 22 great nightshades and 22 carrots) were collected, because the cabbage samples that were to be collected at ONIP Cadjèhoun were missed. Noted that, we were not collected the water samples in wet season, because in this season, the market gardeners don't watered the market garden products. It watered directly by the rain. The samples were collected in sterile Stomacher bags then carried to the laboratory in an icebox at approximately 4˚C.

2.2. Sample Collection of Watering Water

From the four sites listed above, three types of watering water (well, pond and drilling) were collected. The water samples were only collected in dry season (January 2013-February 2013). At each site, three gardeners were randomly selected among those who used the three targeted watering water types, and two samples of each water type were collected per gardener. Twenty-two (22) watering water samples (8 well, 6 pond and 8 drilling) were collected, because the pond water samples that were to be collected at Akpakpa Rigle-Range were missed. All the samples were collected in sterile Stomacher bags and carried to the laboratory in an icebox at approximately 4˚C.

2.3. Microbiological Analysis of Samples

2.3.1. Samples of Market Garden Products
Once at the laboratory, 10 ml of each sample was aseptically poured into sterile bottles and serially diluted with

distilled water up to a 10^{-5} dilution. One milliliter of each dilution (10^{-4} and 10^{-5}) was mixed with 15 ml of plate count agar (~45˚C) and poured in sterile Petri dishes (Olutex Divine Concepts Ltd.). After solidification, a second agar stratum (~4 ml) was added before incubation at 30˚C for 24 h. The colonies grown were counted (30 to 300 colonies per dish).

2.3.2. Samples of Watering Water

Once at the laboratory, microbial analysis of watering water was performed using a membrane filter. After the water samples had been filtered through a filter (HAWP 0.45 μm 0.47 mm), the membrane filter was deposited onto plate count agar poured into Petri dishes and incubated at 37˚C for 24 h. The colonies that grew were counted (30 to 300 colonies per dish), and the values were expressed as Colony Forming Units (CFU)/ml.

2.4. Characterization and Identification of *K. pneumoniae* and Fluorescent *Pseudomonas* spp. Strains

2.4.1. Market Garden Products

K. pneumoniae produces mucoid colonies. They are 4 mm in diameter, curved, shiny, opaque and often confluent. The selective medium EMB (Bio-Rad, French) was then used to select the Gram (-) bacteria. Briefly, 0.1 ml of suspension for each tube dilution (10^{-4} and 10^{-5}) was transferred aseptically into sterile Petri dishes after melting and cooling (45˚C) of Eosin Methylene Blue (EMB) agar. Then, the suspension was spread on the agar surface before incubation at 37˚C for 24 h. The *K. pneumoniae* strains were confirmed using various biochemical tests such as HS_2 and gaz production, lactose and glucose fermentation, mobility, Vogues Proskauer and methyl red, catalase/oxidase/indol/urea production and citrate/mannitol utilization.

The fluorescent *Pseudomonas* spp. strains were isolated on King B medium (Bio-Rad, French). As previously described, 0.1 ml of suspension for each tube dilution was spread on agar and incubated at 30˚C for 24 h. Fluorescent *Pseudomonas* spp. produces green colonies, which is nothing but a characteristic of pyoverdin. Other tests were performed to confirm *Pseudomonas* spp. strains, including the HS_2 and gaz production, lactose and glucose fermentation, assays of mobility, catalase/oxidase/indol/urease/pyoverdin production; citrate/mannitol utilization and lecithin hydrolysis.

2.4.2. Watering Water

For *K. pneumoniae*, a volume of 100 ml of water was filtered on a membrane filter. It was then deposited in the middle of EMB agar previously poured onto Petri dishes and incubated at 37˚C for 24 h. The same method was used for fluorescent *Pseudomonas* spp., but the medium used was King B and the incubation was performed at 37˚C.

2.5. Antibiotic Susceptibility Test for *K. pneumoniae* and Fluorescent *Pseudomonas* spp. Strains

The antibiotic profiles of the isolates were determined using the disk diffusion method on Mueller-Hinton agar (Oxoid, England). Inhibition zone diameter values were interpreted as recommended by the Antibiogram Committee of the French Society of Microbiology [14]. Ten (10) antibiotics (BioMérieux, France) were used in this study: amoxicillin (AMX 30 μg), amoxicillin + clavulanique acid (AMC 20/10 μg), cefotaxim (CTX 30 μg), ceftriaxon (CRO 30 μg), imipenem (IPM 30 μg), tobramycin (10 μg), nalidixique acid (NA 5 μg), ciprofloxacin (CIP 5 μg), triméthroprimsulfamide (SXT 25 μg) and penicillin G (6 μg).

2.6. Phenotypic Detection of Penicillinase Production

The production of penicillinase by the isolated *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains was assessed using the acidimetric tube method [15]. Six hundred (600) milligrams of benzylpenicillin was diluted in 400 μl of distilled water before adding 300 μl of aqueous phenol red solution (1%, w/v). The pH of this solution was then adjusted to 8 with NaOH (1 N). The final 1 ml reaction volume was composed of young *K. pneumoniae* or fluorescent *Pseudomonas* spp. colony suspension and approximately 150 μl of benzyl penicillin solution. The *K. pneumoniae* ATCC 35657 strains were used as a control. A yellow or orange color within 1 h at 37˚C indicated penicillinase activity.

2.7. Phenotypic Detection of Extended Spectrum *β*-Lactamase (ESBL)

The phenotypic detection of ESBL on the isolated *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains was performed using the double disk synergy test [16] [17]. In this test, the tested strains (10^6 bacteria/ml) were flooded onto Mueller-Hinton agar according to the recommendations of the French Society of Microbiology [14]. The test was performed with amoxicillin and clavulanic acid discs and the third-generation cephalosporins, namely, cefotaxime (30 μg) and ceftriaxone (30 μg). The amoxicillin + clavulanic acid disc was placed at the center of the inoculated Mueller-Hinton agar Petri dish whereas the cefotaxime (30 μg) and ceftriaxone (30 μg) discs were placed at both sides (approximately 15 to 20 mm) of the amoxicillin + clavulanic acid disc. After 18 h of incubation at 37°C, the enhancement of the zones of inhibition of any of the cephalosporin disc towards the clavulanic acid disc confirmed the strain to be an ESBL producer [18].

2.8. Detection of Resistance Genes

Polymerase Chain Reaction (PCR) was performed on total DNA from all confirmed ESBL-producer *K. pneumoniae* and fluorescent *Pseudomonas* spp. to detect genes encoding multidrug resistance (TEM, SHV and CTX-M). The DNA template was extracted by suspending a loop of *K. pneumoniae* and fluorescent *Pseudomonas* spp. colonies in 500 μl of sterile, pure water and boiling for 10 min at 95°C. The suspension was then centrifuged for 5 min at 12.000 rpm, and 10 μl of the supernatant was used as target DNA. The latter extracts were stored at −20°C until use.

The primers for bla$_{TEM}$, bla$_{SHV}$ and bla$_{CTX-M}$ were used for multidrug resistance gene investigation by PCR amplification in 30 μL, each containing: 5 μL of DNA, 0.5 μM of each primer (F and R), 1.5 mM MgCl$_2$, 250 μMdNTPs, 1X PCR buffer (Invitrogen) and 1U Taq DNA polymerase (Invitrogen). The PCR program used for amplification consisted of: i-bla$_{TEM}$ (initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min and a final elongation step for 10 min at 72°C), ii-blaSHV (initial denaturation was performed at 96°C for 5 min, 30 cycles at 96°C for 15 s, 50°C for 15 s, 72°C for 1 min and a final elongation step for 10 min at 72°C) and iii-bla$_{CTX-M}$ (initial denaturation was performed at 95°C for 5 min, 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 2 min and a final elongation step for 10 min at 72°C). The primer sequences and the expected fragments are presented in **Table 1**.

PCR products (10 μl) were visualized by electrophoresis at 150 V for 30 min on a 1.5% agarose gel containing ethidium bromide and visualized by UV trans-illumination. A 100-bp ladder standard was used as a molecular weight ladder.

2.9. Data Analysis

The software Microsoft Office Excel 2010 was used to process the data. The software Epi Info 6 (version 6.04cfr, January 1999) was used to perform the Chi-square test. The test was considered significant if $p < 0.05$.

3. Results and Discussion

3.1. Environment of Market Gardening Sites

During this study, many facts were found. On the same site, there are several gardeners grouped together with a head of site. Moreover, some gardeners live on the sites with their whole family in small makeshift huts. Market

Table 1. Primers used to search *bla$_{TEM}$*, *bla$_{SHV}$* and *bla$_{CTX-M}$* genes.

	Taguets genes	Primers	Primers sequences (5' → 3')	Amplicon size (bp)	Reference
Enterobacteria multirestant	*bla$_{TEM}$*	OT-F OT-R	5'-TTGGGTGCACGAGTGGGTTA-3' 5'-TAATTGTTGCCGGGAAGCTA-3'	467	[33]
Enterobacteria multirestant	*bla$_{SHV}$*	SHV-F SHV-R	5'-CGCCGGGTTATTCTTATTTGTCGC-3' 5'-TCTTTCCGATGCCGCCGCCAGTCA-3'	1017	[49]
Enterobacteria multirestant	*Bla$_{CTX-M}$*	CTX-F CTX-R	5'-CGCTTTGCGATGTGCAG-3' 5'-ACCGCGATATCGTTGGT-3'	550	[33]

gardening surroundings are used as a garbage dump not only by the nearby people but also by the growers themselves. Once the vegetable products are harvested, they are sold on the site and women retailers bring them into the markets and other outlets. It is important to emphasize that after the vegetable products have been harvested, they are washed by the same growers in water pools before being routed to the different points of sale. These different practices may be the contamination factors for garden products. These observations are similar to those of Kenmogne [19] and to those of Kifuani [20].

3.2. Microbiological Qualities of Market Garden and Watering Water Products

3.2.1. Density of Mesophilic Microflora

The market garden and watering water products investigated in this study show a varied microbial density (**Table 2**). Among the market garden products, lettuce is more contamined by mesophilic microflora (2.6×10^6 CFU/g) than the other products, apart from carrots, which was slightly less (2.5×10^6 CFU/g). Great nightshade is the least loaded with mesophilic microflora. As far as the watering water types are concerned, the well water contains mesophilic microflora (2.5×10^6 CFU/g) that are clearly more abundant than in the drilling water (1.8×10^6 CFU/g) and pool water (1.5×10^6 CFU/g).

3.2.2. Species of *K. pneumoniae* and Fluorescent *Pseudomonas* spp. Contaminate Market Garden Products and Watering Water

Among the 186 samples collected (market garden products and watering water), 5.91% are contaminated by *K. pneumoniae* and 20.43% by fluorescent *Pseudomonas* spp. Specifically, during the dry season, 5.77% (6/104) and 24.04% (25/104) of market garden products are contaminated by *K. pneumoniae* and fluorescent *Pseudomonas* spp., respectively. In rainyseason, rates relatively decline and 82 samples come from market garden products. Five strains produce *K. pneumoniae*, a rate of 6.1%, and 13 strains of fluorescent *Pseudomonas* spp., a rate of 15.85%. The difference of proportions is significant ($p < 0.05$). The contamination by *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains is more in the garden market products than in the watering water. Nevertheless, there is no significant difference in the rate of contamination of both types of samples (market garden products and watering water). This observation was because the *Pseudomonas* and *K. pneumoniae* species are ubiquitous; they can be found in water, soil, vegetation, sewage and also in the intestinal tract [10] [21]. The presence of strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. in the watering water and market garden products can be explained by the fact that the contamination of vegetable products would be intimately linked to pollution of watering water as stipulated by [22]. In additional, fluorescent *Pseudomonas* spp. is found in rhizosphere of several cultures included the market garden products. Its commonly found bowel moved of human and animals. The fluorescent *Pseudomonas* spp. isolated are *P. aeruginosa* (83.34%), *P. syningae* (8.33%) and *P. putida* (8.33%). In the dry season, three species of *Pseudomonas* are isolated (*P. syringae*, *P. putida* and *P. aeruginosa*), but only *P. aeruginosa* is isolated in the rainy season. The difference of proportions is highly significant ($p < 0.000005$, **Table 3**).

In the dry season, apart from carrots, *K. pneumoniae* and fluorescent *Pseudomonas* spp. are isolated in different proportions from market garden products sampled (cabbage and great nightshade). The lettuce is not contaminated with *K. pneumoniae*, but it is contaminated by fluorescent *Pseudomonas* spp. (23%); the cabbage samples are contaminated by *K. pneumoniae* (9%) and fluorescent *Pseudomonas* spp. (50%) (**Figure 2(a)**). The

Table 2. Mesophilic microflora density contained in market gardens products and water watering.

Samples		Microbial density (CFU/g)
Market gardens products	Lettuce	2.6×10^6
	Carrot	2.5×10^6
	Great nightshade	2.3×10^6
	Cabbage	2.4×10^6
Water watering	Pool	1.5×10^6
	Wells	2.5×10^6
	Drilling	1.8×10^6

Table 3. Market garden products and watering water contamination by *Pseudomonas* species isolated.

	Dry season		Rainy season	Total
	Market garden products	Watering Water	Market garden products	
P. syringae	8.33%	0%	0%	8.33%
P. putida	8.33%	0%	0%	8.33%
P. aeruginosa	38.9%	8.33%	36.11%	83.34%
Total	55.56%	8.33%	36.11%	100%
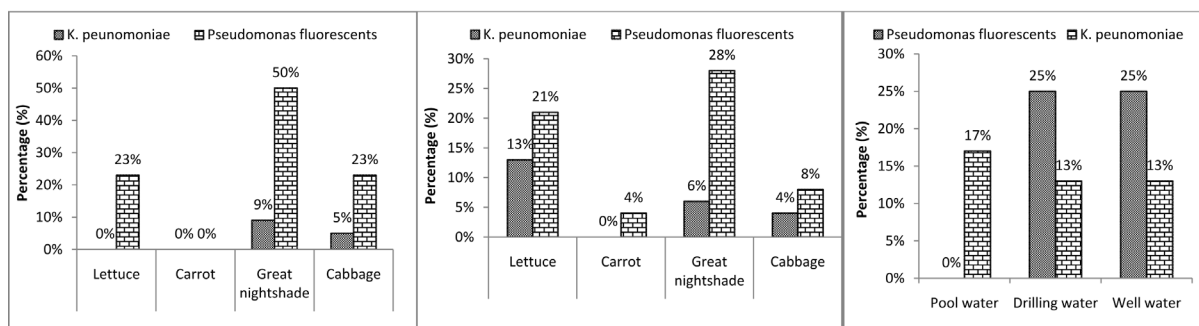
Figure 2. Market garden products and watering waters contamination by *K. pneumoniae* and fluorescents *Pseudomonas* spp. strains.

difference of proportions is significant ($p < 0.05$).

In the rainy season, the four types of market garden products sampled are contaminated. Thus, for fluorescent *Pseudomonas* spp., great nightshade is the most contaminated, at a rate of 28%. It is followed by lettuce (21%), cabbage (8%) and finally carrots that are contaminated by fluorescent *Pseudomonas* spp. (4%). Lettuce is the most contaminated with *K. pneumoniae* (13%); it is followed by great nightshade (6%) and cabbage (4%); carrots are not contaminated with *K. pneumoniae*. The difference of contamination proportions is significant ($p < 0.05$, **Figure 2(b)**). Among the different studied market garden products, and according to the two seasons, carrots are less contaminated than four leafy vegetables (**Figure 1(a)** and **Figure 1(b)**). This result can be explained by the fact that the carrot is a root vegetable in which the edible part is underground. So, it is less exposed to the watering water. Allydice-Francis and Brown [23] showed that lettuce and carrots are the two vegetables mainly associated with *P. aeruginosa* contamination. Only *K. pneumoniae* and fluorescent *Pseudomonas* spp. contamination can be through already-contaminated soils and dirty irrigation water infiltration, because it is recognized that wastewater transports almost all pathogens (bacteria, viruses and parasites) contained in the feces [24]. It is also established that excreta containing pathogens can survive for a long time in the water, soil and plants [25]. Bacterial and viral pathogens can survive on cantaloupe, lettuce and pepper. However, it has been found that, although this contamination occurs by direct contamination of food, the accumulation rates of the strains varies. This observation was made because the variability of the rate of contamination is comparable with the rate from the work of [26].

Considering the three types of water surveyed, well and drilling water are the ones most contaminated by fluorescent *Pseudomonas* spp., at a rate of 25%; drawing water is not contaminated by fluorescent *Pseudomonas* spp. However, pool water is the one most contaminated by *K. pneumoniae*, at a rate of 17%, followed by well and drilling water that is contaminated by *K. pneumoniae* at a rate of 13%. The difference of contamination proportions is not significant ($p > 0.05$) (**Figure 2(c)**).

In the rainy season, lettuce is the most contaminated by *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains (**Figure 2(b)**); it is followed by great nightshade and cabbage. In the dry season, great nightshade is the most contaminated by *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains (**Figure 2(a)**); it is followed by cabbage and lettuce. These observations may find explanation in the morphology of these products and the type of crop. Indeed, [27] asserted that the contamination would be staining direct food at the level of vegetable leaf (cabbage, great nightshade, lettuce, etc.), taking into account the large contact surface, and in the cabbage and

carrot at the same time. The root of vegetables that is in the leaves that close forming a crown, would be a conducive environment for the development of bacteria such as *K. pneumoniae* and fluorescent *Pseudomonas* spp. With regard to great nightshade and lettuce, they have broader leaves, providing a larger surface area for increased contamination by *K. pneumoniae* and fluorescent *Pseudomonas* spp.

In general, vegetables are more contaminated in the rainy season than in the dry season. This observation can explain the presence of water runoff in the rainy season that will drain, directly or indirectly, the feces and all microbes that contain garbage to garden products as supported by [8]. Similar observations were made by [28] in their study on lettuce contamination by *E. coli*.

During the dry season, a variation of rate of contamination is observed depending on the site. Thus, the site in Cadjehoun is the most contaminated one, whereas the site in Akpakpa is the least contaminated one. On the site in Cadjehoun, analyzed vegetable products are contaminated at 4% and 67%, respectively, with *K. pneumoniae* and fluorescent *Pseudomonas* spp. (**Figure 3(a)**); at the same site, watering water analyzed is contaminated with *K. pneumoniae* at 17% and 33% by fluorescent *Pseudomonas* spp. As for the site in Akpakpa, all watering water is not contaminated, while the vegetable products are contaminated at 6% by fluorescent *Pseudomonas* spp. The difference of contamination proportions is significant ($p < 0.05$) (**Figure 3(c)**). During the rainy season, the results are based on the analysis of the vegetable products. Thus, on the site in Cadjehoun, the contamination rate is 11% for each of the two types of strains. The Site of Akpakpa seems to be the most contaminated one this season, with a rate of contamination of 4% and 25%, respectively, for *K. pneumoniae* and fluorescent *Pseudomonas* spp. Meanwhile, the site of Houéyiho-Barrier is contaminated by fluorescent *Pseudomonas* spp. at a rate of 13%. The difference in proportions is significant ($p < 0.05$, **Figure 3(c)**).

3.3. Susceptibility of Strains to Antibiotics

All of the 11 strains of *K. pneumoniae* isolated are resistant to amoxicillin and penicillin. Greater than 50% resistance rates are observed with amoxicillin + clavulanic acid (82%), 64% with Trobamycin and finally 55% with Ceftriaxone (**Table 4**). Forty-five percent of strains are resistant to trimethoprim sulfa; 36% to nalidixic acid and only 9% to ciprofloxacin. None of the strains are resistant to imipenem. The difference of resistance proportions is significant ($p < 0.05$). All of the thirty-eight (38) strains of fluorescent *Pseudomonas* spp. are resistant to cefotaxin, amoxicillin + clavulanic acid, ceftriaxone, amoxicillin, nalidixic acid and penicillin. 82% of the strains are resistant to trimethoprim sulfa; 47% to trobamycin and only 8% of these strains to ciprofloxacin. None of the fluorescent *Pseudomonas* spp. strains isolated were resistant to imipenem. The difference of resistance proportions is significant ($p < 0.05$).

The *K. pneumoniae* strains are resistant to many antibiotics tested. It is well-known that the *β*-lactamines are the class-one antibiotics for the treatment of *K. pneumoniae* infections in adults and children [29] [30]. Some research confirms the natural resistance of *K. pneumoniae* to amoxicillin, ampicillin and carboxyenicillines [10] [31]. So, in this study, all *K. pneumoniae* strains are resistant to amoxicillin and penicillin (100%), while 82% resistance to amoxicillin + clavulanic is noticed. This rate is higher than the one obtained in Algeria [32]. These studies of clinical strains reported a rate of 52.4% for resistance to amoxicillin + clavulanic acid. *K. pneumoniae* strains are resistant at 9% and 36%, respectively, to ciprofloxacin and nalidixic acid. These results are higher
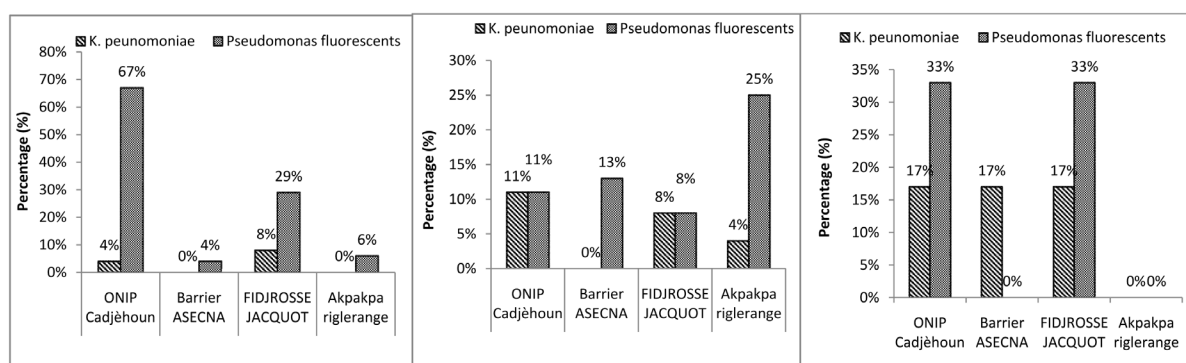

Figure 3. Distribution of different strains of *K. pneumoniae* and fluorescents *Pseudomonas* spp. depending on the sites.

Table 4. Antibiotics profile of *K. pneumoniae* and fluorescents *Pseudomonas* spp. strains isolated.

Antibiotics	Resistance (%)	
	K. pneumoniae (n = 11)	fluorescents *Pseudomonas* (n = 38)
CTX	55	100
AMC	82	100
CRO	55	100
AMX	100	100
TM	64	47
CIP	9	8
IPM	0	0
NA	36	100
SXT	45	82
P	100	100

Amoxicillin (AMX), amoxicillin/clavulanicacid (AMC), cefotaxime (CTX), ceftriaxone (CRO), imipenem (IPM), tobramycin (TM), nalidixic acid, (NA), ciprofloxacin (CIP), and trimethoprim/sulfamethoxazole (SXT) and penicillin (P). n = number of strains.

than those reported by [31], who found resistance rates of 0% for ciprofloxacin and 35% for nalidixic acid in *K. pneumoniae* from all types of clinical specimens. In general, in this study, the rates of resistance that we have seen are very high for most antibiotics compared with those observed in industrialized countries, but similar resistance rates are reported in some developing countries [33]. This high rate of resistance could be explained by the excessive and uncontrolled use of these antibiotics by the populations, especially in developing countries; that would result in the acquisition of factors of resistance to antibiotics by microorganisms [34] [35].

The susceptibility results of fluorescent *Pseudomonas* spp. strains to antibiotics show that they are resistant to the vast majority of the tested antibiotics. The rates of resistance to the trimethoprim/sulfamethoxazol and ciprofloxacin are, respectively, 82% and 8%. This result is comparable to the 83% and 7% rates observed by [23]. Tobramycin resistance is 47%; this rate is higher than the one found by [36], who found a rate of resistance to tobramycin of 34.4%.

In this study, all of the strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. were not resistant to imipenem. The fluorescent *Pseudomonas* spp. isolated from fresh vegetables by [23] are not resistant to imipenem (0%). This result demonstrates that imipenem would remain the most active molecule on *K. pneumoniae* and fluorescent *Pseudomonas* spp. This rate is lower than the one found by [37] in Tunisia who have found 19.6% resistance to imipenem. Indeed, [38] studied the emergency risk of resistance associated with piperacillin, ceftazidime, ciprofloxacin and imipenem observed by a variable emergency risk, with maximum resistance to imipenem ($p < 0.001$).

For *K. pneumoniae*, this rate is comparable to the 0.4% observed by [39] in the food sector and confirmed by the 0% to 2% rate of resistance observed by [10] in their study of some patients hospitalized in Europe and in the United States of America. However, this rate is different from the 16% observed by [40] in Cameroon on clinical strains and the 10% observed by [41] in America in the same field. Thus, the difference can be explained by the origin of the involved strains, although the strains have acquired resistance against antibiotics. This resistance is due to improper and uncontrolled use of antibiotics. This high rate of resistance in this study could be explained by the misuse of the antibiotics by the population in our country. Microorganisms would therefore have time to develop resistance to antibiotics. On the other hand, the strains come from the food raw materials that have not yet faced the misuse of antibiotics.

3.4. Penicillinase and ESBL Production by Isolated Strains

The research on penicillinase reveals that 22% of the *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains produce penicillinase. The 11 strains producing penicillinase are composed of 45% of *K. pneumoniae* and 55% of fluorescent *Pseudomonas* spp. strains. The difference in proportions is significant ($p < 0.05$). None of the isolated strains of *K. pneumoniae* and fluorescent *Pseudomonas* spp. contained expanded spectrum *β*-lactamase-

producing plasmids (ESBLs).

3.5. Presence of Encoding Gene for Penicillinase Production by Strains

The DNA of those strains is used to seek either the presence or the absence of the bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. The compilation of this investigation (**Figure 4**) first displays that 64.29% of the tested strains carry the bla_{TEM} gene, 21.43% carry the bla_{SHV} gene and finally 14.28% carry the bla_{CTX-M} gene (**Table 5** and **Figure 4**). Only the strains isolated in the rainy season carry the bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. The distribution of the *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains considerably varies according to the season. The difference of proportions is significant ($p > 0.05$).

Neither isolated *K. pneumoniae* nor fluorescent *Pseudomonas* spp. produce ESBLs, whereas the genes bla_{TEM}, bla_{SHV} and bla_{CTX-M} were present in some of *K. pneumoniae* and fluorescent *Pseudomonas* spp. strains isolated in this study. This observation can be explained by the fact that, with some species, the natural resistance of β-lactamines is binding to the component production of SHV that is the resistance to penicillins at the smaller level of cephalosporins of first generation [42] [43]. No strains in the dry season have the genes bla_{TEM}, bla_{SHV} and bla_{CTX-M}. Their presence in the rainy season can be explained by the bad weather and more microorganisms developing a method of resistance in the presence of these genes. In the clinic, the higher rate reached 95.45% for the presence of the bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes observed in the *K. pneumoniae* produced by ESBLs in some studies [44]-[47]. This difference in proportion is because; the strains in this study come from food samples. However, these strains are very dangerous for humans when they ingurgitate, because they have developed multiple resistance that leads to the complications observed in a patient in the hospitals. The infections induced by these strains represent a high risk of therapeutic failure, and furthermore, they are associated with long-term hospitalization and consequently higher hospital expenses [48].
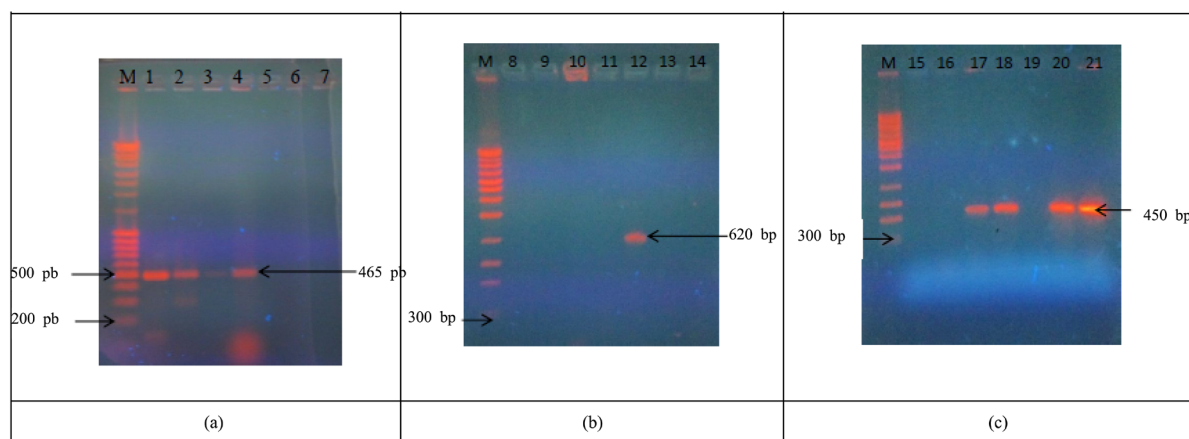

Figure 4. Detection of the presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes.

Table 5. Distribution of the pencillinase genes carried by strains of *K. pneumoniae* and fluorescents *Pseudomonas* spp.

| | Dry season | | | | Rainy season | | |
| | Market garden products | | Water of watering | | Market garden products | | |
	K. pneumoniae (n = 3)	Flurescents *Pseudomonas* (n = 22)	*K. pneumoniae* (n = 3)	Flurescents *Pseudomonas* (n = 3)	*K. pneumoniae* (n = 5)	Flurescents *Pseudomonas* (n = 13)	Total
Bla_{TEM}	0%	0%	0%	0%	28.58%	35.71%	64.29%
Bla_{SHV}	0%	0%	0%	0%	14.29%	7.14%	21.43%
Bla_{CTX-M}	0%	0%	0%	0%	7.14%	7.14%	14.28%
Total	0%	0%	0%	0%	50.01%	49.99%	100%

n = number of strains.

4. Conclusions

The importance of fruits and vegetables in human health was admitted. The slogan of five fruits and vegetables per day is known in the whole world. Paradoxically, owing to the lack of information and hygiene, most cases of food contamination are caused by the same fruits and vegetables. It is a major problem for the population. This study was therefore designed to inform the population about the biochemical and molecular strains of *Klebsiella pneumoniae* and fluorescent *Pseudomonas* spp. that were very harmful to human health and isolated from watering water and vegetable products in Cotonou (Benin) during different seasons.

This study has allowed for biochemical characterization of *K. pneumoniae* and fluorescent *Pseudomonas* spp. isolated from watering water and vegetable products and the determination of their antibiotic resistance profile. Moreover, after some investigations and various analyses, we found that different watering water used for vegetable products was not suitable, which might explain the strong presence of infectious diseases such as gastroenteritis, typhoid fever, cholera, etc. in Benin. It is therefore important that the authorities might make growers aware of the risks related to their practices. In order to prevent or decrease microorganisms pollution in natural food and watering water, the followed measures should be payed: To treat the watering water before use, to treat the composts used to fertilize the market garden products, because it contains several pathogenic microorganisms, to keep the production sites of market garden products salubrious (don't shat in site, to prevent access of site by animals, to put the household refuse out the site), at end to wash correctly the market garden products before to eat.

Acknowledgements

Author's Contributions

Both authors shared in the preparation of the manuscript. LB-M, TAA and WM designed the study. MGT and WM collected data. NWC, PAN and HS analyzed data and interpreted the results. WM and PAN drafted the manuscript. LB-M, TAA, NWC and HS revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Declaration of Interests

The authors declare there are no competing interests about the publication of this manuscript.

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