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MOLECULAR DETECTION OF SALMONELLA SPECIES FROM SELECTED VEGETABLES SOLD IN A NORTH-CENTRAL NIGERIAN SETTING

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ABSTRACT

It is vital to study and understand the genetic basis to the virulence of different *Salmonella* strains in order to fully grasp the facts behind the unique capabilities of these pathogenic agents to causing diseases in both humans and animals. In this study, the conventional microbiological culture methods were used to isolate pure *Salmonella* strains from 120 vegetable samples of five different types; which were all obtained at seven different popular markets in the Jos Metropolis of North-Central Nigeria. 25 (20.8%) pure isolates were obtained from 120 samples after initial culture and sub-cultures; with 24 (20%) of the pure isolates testing positive as being pathogenic after biochemical analysis. From the 25 pure isolates, the same 24 which tested positive for biochemical tests were also successfully amplified by PCR technique with the *Salmonella invA* virulence gene. The result shows that 96% of the pure isolates were positive for the *Salmonella invA* gene. The PCR product which was very specific is a 250bp fragment of DNA which was visualized in 1.5% agarose gel. This finding shows that virulent *Salmonella* strains pose a major health hazard and public health concern to the affected population. Our study shows that there is a high prevalence rate of virulent *Salmonella* strains in North-Central Nigeria. It is thus concluded that although both the conventional culture and biochemical methods of isolating *Salmonella species* are most useful for obtaining pure isolates and identifying pathogenic strains, however, the PCR technique remains the most specific and sensitive; especially when the rapid identification and detection of virulent strains of *Salmonella species* are of utmost importance.

Key words: Virulence, *invA* gene, PCR, North Central Nigeria

LA DETECTION MOLECULAIRE DES ESPECES DE SALMONELLES DE LEGUMES SELECTIONNES VENDUS AU NORD CENTRAL D'UN CADRE DU NIGERIA

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RESUME

C'est important d'étudier et comprendre la base génétique de la virulence des souches différentes de *Salmonelles* afin de saisir pleinement les faits derrière les capacités de ces agents pathogènes à l'origine de maladies chez les humaines et les animaux. Dans cette étude, les méthodes classiques de culture microbiologiques ont été utilisées pour isoler des souches de *Salmonelles* pures de 120 échantillons de légumes de cinq types différents ; qui ont été obtenus aux sept marchés populaires différents dans la métropole de Jos au nord - central du Nigeria. 25 (20,8%) isolats purs ont été obtenus de 120 échantillons après la culture initiale et la sous - culture ; dont 24 (20%) des isolats purs ont été testés positifs comme pathogènes après l'analyse biochimique. De 25 isolats purs, les mêmes 24 qui ont été testés positifs pour des tests biochimiques ont également été amplifiés par la technique PCR avec succès avec le gène de virulence de *Salmonelle invA*. Le résultat montre que 96% des isolats purs étaient positifs du gène de *Salmonelle invA*. Le produit de PCR qui était très spécifique est un fragment de 250bp de l'ADN qui a été visualisé en 1,5% gel d'agarose. Ce résultat montre que les souches de *Salmonelles* virulentes présentent un risque majeur pour la santé et le souci de la santé publique à la population touchée. Notre étude montre qu'il y a un taux élevé de prévalence de la virulence des souches de *Salmonelles* dans le centre - nord du Nigeria. Il est donc conclu que, bien que la culture conventionnelle et les méthodes biochimiques d'isoler des espèces de *Salmonelles* sont les plus utiles pour l'obtention des isolats purs et identifier les souches pathogènes, cependant, la technique PCR reste la plus spécifique et sensible ; en particulier lorsque l'identification rapide et la détection de souches virulentes d'espèces *Salmonelles* sont d'une importance capitale.

Mots clés : Virulence, le gène *invA* , PCR, Centre - nord du Nigeria.

INTRODUCTION

Salmonella is a bacterium of the genus *enterobacteracea*. There are many strains of this group of bacteria [1, 2]; with many commonly found in the environment, while some others have specific animal hosts. Salmonellosis is a generally and commonly distributed food borne disease all over the world [1, 3, 4]. Salmonella infection and thus Salmonellosis is acquired through contaminated animal products, contaminated water, contaminated fruit and vegetables, and also via the feco-oral route through person-to-person contact [1, 3, 4, 5]. Salmonellosis is usually characterized by abdominal cramp, diarrhea, fever, headache, nausea and vomiting among others [2, 3, 4]. Usually, individuals with compromised immunity, the geriatrics, and pediatrics; especially children under five years of age are the most prone to this disease [2, 3, 4]. Complications associated with Salmonellosis include loss of body fluid (electrolytes); due to persistent diarrhea which may lead to death if not well managed. Also, 'Reactive Arthritis' may result from *Salmonella* infection [4].

Globally, several millions of people are infected with *Salmonella species* annually [1]; and with many strains of this bacterial now developing resistance against antimicrobial agents, Salmonellosis has thus become a disease of major Public Health concern [1,17]. There are different serotypes of *Salmonella species*; but the two strains which are both zoonotic and pathogenic, and most common worldwide are the *Salmonella typhimurium* and *Salmonella enteritidis* [1].

Studies have shown the prevalence of *Salmonella species* in different poultry and animal products [6, 7]. Some studies in Nigeria [8, 9, 10] and at different parts of the world [11, 12, 13, 14] have indicated the pollution and or contamination of most common vegetables by *Salmonella species*. In North-Central Nigeria where the occupation is predominantly farming, most small scale farmers cultivate vegetable farms; and the produce from these farms are generally marketed at the different markets within the respective local communities. The cultivated vegetables are mostly watered manually with contaminated waters from nearby streams or water bodies. These farms are mostly manured with infected and or contaminated poultry droppings and animal dung [15, 16] which serves as a rich source of nitrogen to the growing crops.

Conventional culture methods for the growth and isolation of bacteria are at best most useful for advanced molecular diagnostics and biochemical identification of pathogenic strains. The use of polymerase chain reaction (PCR) serves as the most reliable method of identifying and detecting pathogenic *Salmonella species* and their virulence gene.

MATERIALS AND METHODS

This study was carried out in Jos, the capital of Plateau State, North-Central Nigeria. The Jos Metropolis comprises of three different local government areas; namely, Jos North, Jos South, and Jos East local governments respectively. The occupation of the people on the Jos Plateau is predominantly farming. This farming is largely encouraged by the naturally fertile soil which enhances the growth of most vegetables and food crops. The seven major markets in the Jos Metropolis were visited for the sampling of the various vegetables used in this study. These markets are as follows: Gadabiu market, Terminus market, Faringada market, Gyel market, Bukuru market, Building Materials market, and the Angwan Rukuba market. The selected vegetables for the study include Cabbage, Carrot, Lettuce, Garden egg, and Cucumber respectively. All samples were aseptically collected into sterile poly bags, and adequate precautions were taken to avoid human contamination before transportation to the Laboratory for testing. A total of 120 samples of the above listed vegetables were randomly selected and used in this study.

CULTURE ISOLATION OF SALMONELLA BACTERIA

All the media used in this study, which includes Peptone water, Xylose Lysine Deoxycholate agar, Salmonella-Shigella agar, Brain-Heart Infusion media, and Nutrient agar, were all prepared according to methods as described by Ochei *et al* [18]; and they were all sterilized at 121°C for 15 minutes; except for the *Salmonella-Shigella* agar which was boiled for 15 minutes as this does not require autoclaving. All samples collected were aseptically swabbed and cultured; and incubated at 37°C for 24 hours in the selected medium until pure isolates were identified and biochemically tested by the Urease test, Simmons Citrate test, and the Andrade's Peptone sugar test[18]. The pure *Salmonella* isolates which serves as stock were refrigerated at 2°C - 8°C and then used for DNA - PCR analysis.

SALMONELLA DNA EXTRACTION

1.5mls Eppendorf tubes were labeled based on the number of pure isolates obtained; which was 1 to 25, and these were well arranged on a rack. 1ml of the pure isolates suspended in Brain-Heart infusion broth was then dispensed into the 1.5ml already labeled Eppendorf tubes. This was centrifuged at 14,000 rpm (rate per minute) for 3 minutes using the Eppendorf Cold Centrifuge until a supernatant and pellets are obtained - this process is known as 'Cell Washing'. The supernatant was discarded, and the pellet was adjusted to 100µl using normal phosphate buffered saline with the aid of a micropipette. This was vortexed and properly dissolved. 5µl of the digestion buffer was then dispensed into each of the Eppendorf tubes. A further 5µl of Proteinase K was also added to the buffer and this was well vortexed to aid the even distribution of the added buffer and enzyme. The mixture was then incubated at 55°C for 20 minutes at the end of which the mixture appears transparent. 70µl of the genomic lysis buffer was then added to the mixture and this was thoroughly mixed.

The mixture was transferred to a Zymospin mini spin column tube and then centrifuged at 12,000 rpm for 1 minute. 200µl of DNA pre-wash buffer was added to the spin column and again centrifuged at 12,000 rpm for 1 minute. To enable the absolute washing away of all debris, 400µl of DNA wash buffer was dispensed into the spin column and centrifuged at 12,000 rpm for 1 minute. The mixture was then transferred to a clean micro-centrifuge tube where ≥50µl of DNA elution buffer was added. The tube was allowed to stand at room temperature (RT) for 2 to 5 minutes before being centrifuged at top speed for 30 seconds in order to elute the DNA. The eluted DNA was then taken for amplification reaction in an Eppendorf Thermocycler.

PCR AMPLIFICATION AND PRIMER SETS

A reverse and forward specific *Salmonella invA* primer of 250bp was used for the PCR of our quantified pure DNA extracts. This specific *Salmonella invA* gene has the following bases: 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA - 3' and 5' - TCA TCG CAC CGT CAA AGG AAC C - 3' respectively. The amplification mixture consist of the 'Master Mix' which is made up of 3µ of 10X PCR buffer, 1µl of MgCl₂, 1µl of dNTPs (10Mm), 1.25µl each of *invA Forward and invA Reverse polymerase*. 5µl of the sample was added,

and the total volume of the mixture was made up to 25µl.

PCR amplification was carried out in an Eppendorf Master-Gradient Thermocycler. The stages of amplification involves: incubation at 94°C for 60 seconds, followed by 35 cycles of denaturation at 94°C for 60 seconds, this is followed by annealing at 64°C for 30 seconds, and elongation of the annealed primers at 72°C for 30 seconds; finally, there was the extension of the amplified sequences at 72°C for 7 minutes.

AGAROSE GEL ELECTROPHORESIS

The amplified DNA products from the *Salmonella* specific PCR were analyzed on 1.5% w/v agarose gel; stained with ethidium bromide. 10µl of each amplified product was mixed with 2µl of 6X loading dye and then loaded onto the agarose gel. A 250 base pair (bp) ladder was used as a marker for the PCR product; and a current of 120volts for 30 minutes was passed through the electrophoretic tank. The amplified products for the positive control which was obtained from animal source yielded result at the expected band size of 284bp; while the pure isolates from the plant sources yielded base pairs of varied bands, with fourteen of the pure isolates yielding a consistent base pairs of 260.

RESULTS

This study was carried out in order to shed more light on the *Salmonella species* contamination of commonly available vegetables; and the danger this contamination poses to the public health of the affected populations. Of the 120 samples analyzed from the seven selected markets in the Jos Metropolis, 25 samples were concluded pure isolates after repeated cultures and sub-cultures. But with biochemical analysis, 24 of the pure isolates tested positive for pathogenicity. The DNA of the pure isolates which were re-suspended in Brain-Heart infusion broth was extracted and used for PCR. The percentage prevalence of the cultured and sub-cultured pure isolates was 20.8% representing 25/120 samples; while the percentage prevalence of those isolates which were biochemically positive and also successfully amplified with the virulent *invA* Forward and Reverse *Salmonella* gene was 20% which represents (24/120) samples. 24 of the 25 pure isolates tested positive for *Salmonella invA* specific gene and this represents a percentage of 96%; which clearly indicates a high prevalence rate of the virulent strains of *Salmonella species* in

this study. This prevalence rate poses a very severe danger to the public health of the affected population; thus affecting their socio-economic

and general wellbeing (Table 1; and Figures 1 and 2).

TABLE 1: SUMMARY OF SALMONELLA SPECIES ISOLATES FROM VEGETABLES SOLD IN SEVEN SELECTED MARKETS IN THE JOS METROPOLIS OF PLATEAU STATE, NORTH-CENTRAL NIGERIA.

Market		Cabbage	Carrot	Lettuce	Garden egg	Cucumber	Total
Jos North	2	1	2	1	2		8
Jos South	2	1	2	1	1		7
Jos East	4	4	1	0	1		10
Total	8	6	5	2	4		25

FIGURE 1: PREVALENCE OF SALMONELLA SPECIES IN THE EXAMINED VEGETABLES.

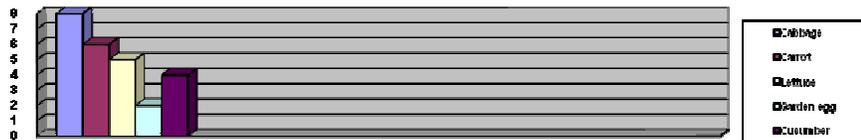
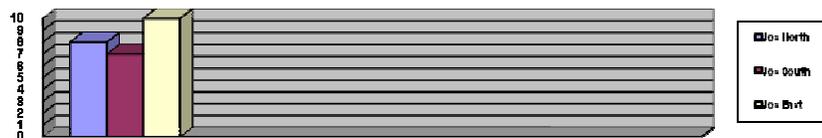


FIGURE 2: PREVALENCE OF SALMONELLA SPECIES IN THE THREE LOCAL GOVERNMENT AREAS OF THE JOS METROPOLIS IN PLATEAU STATE, NORTH-CENTRAL NIGERIA.



DISCUSSION

The repeated bouts of *Salmonella* infections, especially with the non-typhoidal *Salmonella* strains has become an issue of public health concern worldwide [1]. Though most cases of Salmonellosis are usually mild, this disease may sometimes lead to fatal outcomes [2, 4]. Poor hygiene practices; such as inadequate washing of hands, fruits and vegetables, contaminated food and water, and contaminated animal products, are all responsible for the transmission of

Salmonellosis [1, 3, 4]. Also, with the rapid emergence of antimicrobial resistance strains of *Salmonella species* [17], it is of utmost importance that healthy individuals do not take routine antimicrobial medications in mild to moderate cases; safe in geriatrics, infants or children below five years of age, and immune-compromised individuals [1].

Safe ways of handling fruits and vegetables before personal and or public consumption have been well enumerated and outlined by various public health authorities and cooperate organs [1, 19, 20]; and now, the onus lies on the individuals to safeguarding their personal and thus, the health of the public through the strict observance and adherence to the universal and basic rules of hygiene and food safety.

Different studies have shown very high prevalence rate of *Salmonella* contamination of vegetables [8, 14] and different animal products [6, 21, 22]. The prevalence rates from these studies compares favorably with the 20% of the 120 study samples and the 96% of the 25 pure *Salmonella* isolates of DNA-PCR *Salmonella invA* gene detectable results obtained in this current study. The high virulence rate of 96% obtained in this study however poses a severe health risk to the affected populations. The observed contamination of the studied vegetables in this study emerged most probably as a result of either the manure (poultry droppings or animal dung) used to grow the crops; or the water sources used for the irrigation of the crops; which in most instances harbors the pathogen. Our study reveals that though the use of the conventional culture methods are most useful for obtaining pure *Salmonella* isolates, and the application of biochemical tests helps in identifying pathogenic strains; which is indeed most vital in poor resource settings and especially in low and middle-income countries where PCR technique materials may not be readily available. However, the PCR technique remains the most specific and the most sensitive; especially when the rapid identification of virulent strains of *Salmonella species* are of utmost importance.

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CONCLUSION

25 (20.8%) pure culture isolates were obtained in this study; out of a total of 120 samples. 24 (96%) of the pure isolates tested positive for the *invA Salmonella* virulence gene. This indicates a very high prevalence rate of virulent *Salmonella* strains in the study area. And though the rich and fertile nature of the soil on the Jos Plateau, North-Central Nigeria favors the growth of very large varieties of vegetable crops, the use of contaminated poultry droppings and animal dung as manure, and the use of contaminated water sources for the irrigation of the farm lands, coupled with poor personal hygiene practices, have all led to the increased reports in the cases of Salmonellosis. It is therefore advised that urgent public health steps be taken by concerned authorities towards public education on food safety, observance of basic personal hygiene like hand washing, and information on known sources of *Salmonella* contaminants, so as to foster a healthy and a productive society through awareness and the observance of basic preventive measures.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All authors participated in this study. The final manuscript was well read and approved by all authors.

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