International Journal of Microbiology and Biotechnology
2018; 3(3): 79-82
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20180303.13
ISSN: 2578-9678 (Print); ISSN: 2578-9686 (Online)

Detection of *invA* Gene in *Salmonella* Limete Isolated from Wastewater Treatment Plant of University of Nigeria Nsukka, Nigeria

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To cite this article:
Anthony Chibuogwu Ike, Dickson Ihenrochi Dickson, Okechukwu John Obi. Detection of *invA* Gene in *Salmonella* Limete Isolated from Wastewater Treatment Plant of University of Nigeria Nsukka, Nigeria. *International Journal of Microbiology and Biotechnology.* Vol. 3, No. 3, 2018, pp. 79-82. doi: 10.11648/j.ijmb.20180303.13

Received: October 1, 2018; **Accepted**: October 22, 2018; **Published**: November 10, 2018

Abstract: Salmonellae are ubiquitous microorganisms that infect both humans and animals. Human infections usually occur through contaminated food or water and can result in one of two major diseases, namely gastroenteritis and enteric fever. Hence, *Salmonella* remains a major public health problem especially in developing countries where the level of hygiene is very low. The objective of this study was to evaluate the potential risk of *Salmonella* serovars isolated from the University of Nigeria Nsukka (UNN) wastewater treatment plants. Three *Salmonella enterica* subspecies *enterica* serovar Limete isolates from the UNN waste treatment plants were investigated for the presence of invasive A (*invA*) gene. Deoxyribonucleic acid (DNA) was extracted from the isolates by boiling method. Extracted bacteria DNA was amplified by polymerase chain reaction (PCR) using *invA* specific primers. PCR products were resolved on 1.5% agarose gel stained with 0.5 µg/ml of ethidium bromide. Results showed the presence of a band size of 244 base pair of *Salmonella invA* gene in 2 of the isolates. This is an indication that the isolates may have a human or animal origin and are potentially pathogenic. Therefore, the treatment of water in the wastewater plant is insufficient and water from the plant should not be employed for human use or used with caution.

Keywords: *Salmonella Enterica*, *InvA* Gene, Wastewater, DNA, PCR, Serovar

1. Introduction

Salmonella species have been frequently isolated from wastewater, which are frequently reused in food production in different parts of the world [1, 2]. *Salmonella* are known to cause severe disease symptoms that range from self-limiting diarrhoea to bacteraemia. They are the etiological agents of a wide range of diseases such as salmonellosis and typhoid fever and are among the leading causes of gastroenteritis worldwide [3, 4].

Salmonella enterica is the only species of *Salmonella* with medical importance. The species *S. enterica* is taxonomically divided into six subspecies and contains over 2,600 serovars, of which subspecies I serovars account for more than 99% of all *Samonella* infections in humans. The subspecies I can be divided into two clinically important groups, those that cause life-threatening systemic infections known as typhoid or enteric fever and the non-typhoidal serovars that cause self-limited gastroenteritis in humans [5]. One of the main sources of the typhoidal servars in endemic areas, such as Nigeria, is consumption of contaminated water or foods prepared with such contaminated water [6].

In many parts of the world, water supplies for domestic consumption, agriculture and industrial uses are no longer able to keep up with demand. Water reuse is becoming more commonly considered as a viable option for addressing these needs. Indirect reuse of wastewater have been reported in many places and this has resulted in increased incidences of waterborne diseases with far reaching socio-economic and public health implications; thus water quantity and quality issues are both of concern [7].

Despite large advances in water and wastewater treatment, waterborne diseases still pose a major threat to public health worldwide. The presence of microbial pathogens in polluted,

untreated and treated water presents a considerable health risk to both humans and animals [8]. Furthermore, some researchers have reported that the conventional wastewater treatment do not guarantee the complete elimination of many pathogens [9, 10].

Invasiveness is one of the virulence factors of Salmonella. *InvA* and other related genes are known to be responsible for the invasion of epithelial cells in *Salmonella* [11]. *InvA* is contained in the *Samonella* pathogenicity island (SPI), which carries the major components required by *S. enterica* to cause infections [12]. The *invA* genes are contained in SPI-1 [13], which has been found to be absent in environmental *Salmonella* isolates but present in invasive isolates of the same serovars isolated from mammals [14].

This study reports the detection of *invA* gene in *S. enterica* serovar Limete isolated from wastewater treatment plant of UNN, Nigeria.

2. Materials and Methods

2.1. Isolation and Identification of Salmonella

Salmonella species were isolated from wastewater samples collected from the wastewater treatment plant of the University of Nigeria, Nsukka (UNN) and identified according to the standard methods for the examination of water and wastewater described by ISO [15] and APHA [16] as already reported [17].

The isolates were confirmed and serotyped based on slide seroagglutination using commercially available *Salmonella* polyvalent O, H and Vi antisera (Difco, USA) based on White-Kauffmann-Le Minor classification scheme. This was carried out at WHO Collaborating Centre for Reference and Research on *Salmonella* at Pasteur Institut, France.

2.2. Preparation of Genomic DNA

Bacterial DNA was extracted by boiling according to the method described by Medici *et al*. [18] as already reported [17]. Extracted DNA was stored at 4^{o}C until used as a template for amplification.

2.3. Polymerase Chain Reaction (PCR)

The *invA* gene was amplified by an established PCR technique [20], using the INVA primers (Table 1).

Table 1. *Primer sequence and reaction parameters.*

Primer	Sequence (5'–3')	Gene	Corresponding position	Reference
INVA-1	ACA GTG CTC GTT TAC GAC CTG AAT	*invA*	104 - 127	Chiu and Ou [19]
INVA-2	AGA CGA CTG GTA CTG ATC GAT AAT	*invA*	324 -347	

Amplification was carried out in a 25 µl total volume of PCR mixture containing 2 µl of template DNA, 4 µl of the PCR Master Mix (Solis BioDyne, Estonia) (1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1U Taq Polymerase), 20 pmol of each primer and ddH_20 to the total volume of 25µl. DNA amplification was done in an Eppendorf vapo protect thermocycler (Hamburg, Germany). Amplification was performed with the following cycling profile: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 2 min; and a final extension of 72°C for 7 min. The amplified DNA fragments were resolved by gel electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.5µg/ml) and visualized with a photogel documentation system (Cjinx Science Instrument, USA). A positive control (DNA from *S*. Typhi) and a negative control (sterile distilled water) were included in the PCR.

3. Results

The UNN treatment plant is made up of 2 Imhoff tanks A and B and 2 waste stabilization ponds (WSPs) A and B. Twenty-five isolates each from the 2 Imhoff tanks and the 2 WSPs were investigated. Out of the 100 isolates, 18, 16, 16 and 15 from Imhoff tank A, Imhoff tank B, WSP A and WSP B respectively were found to be urease negative. Out of the 12 presumptive *Salmonella* isolates identified from the 65 isolates after biochemical testing, 3 were confirmed as *S. enterica* serovar Limete. Two of the *Salmonella* Limete isolates came from samples collected from Imhoff tank A, while the remaining isolate came from WSP A (Table 2). Although the WSPs are divided into A and B, there is no complete division separating them and materials move between them.

Table 2. *The number of isolates from the different parts of UNN wastewater treatment plants.*

Parameter	Imhoff Tank A	Imhoff Tank B	WSP A	WSP B
Total no. of isolates	25	25	25	25
Urease negative	18	16	16	15
Presumptive *Salmonella* isolates	5	3	3	1
S. enterica serovar Limete	2	0	1	0

The result of the *invA* PCR assay revealed that 2 of the 3 *S. enterica* serovar Limete isolates were found to harbor the *invA* gene corresponding to 244 bp (Figure 1).

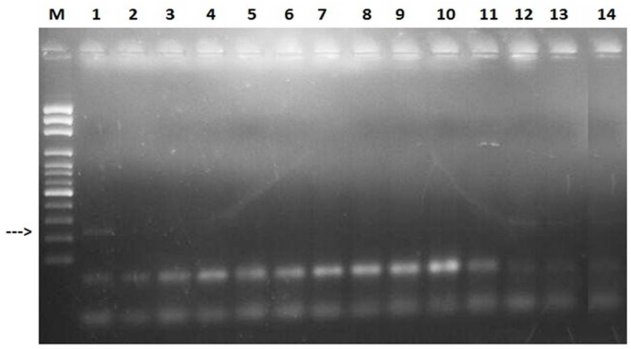

Figure 1. *Agarose gel electrophoresis showing amplification of 244 bp fragment of invA gene in Lane 12 and 13. Lane M: PCR Marker; Lane 1: Positive Control; Lane 2: Negative Control; Lane 3-14: samples.*

An exception was observed in the case of one isolate which did not show the presence of the *invA* gene. All non-*Salmonella* samples gave negative results.

4. Discussion

Isolation of *Salmonella* from wastewater is common, as is also the reuse of treated water for food production [1, 2]. This practice is also carried out by the community living around the UNN wastewater treatment plants. They often use water from the treatment plants for irrigation of vegetables. Such vegetables are then harvested and sold in the market.

Salmonella enterica serovar Limete is not a typical human pathogen and is rarely associated with human illness [21]. The isolation of this serovar from wastewater treatment plant is not unexpected as wastewater often contains strains from other sources outside of humans, including those from animal origin [10, 22]. The presence of *S*. Limete was reported in water samples from 3 different farms in Gran Canaria, Spain [23]. Also, the serovar has also been recovered from human faeces in Sudan [24]. This broad host spectrum is a strong indication that contamination of the wastewater may be of either human and/or animal origin.

PCR assay was used to detect *Salmonella* spp targeting the *invA* virulence gene. The result of the invA PCR assay revealed that 2 of the 3 *Salmonella* isolates previously identified as *Salmonella* Limete were found to harbour the *invA* gene corresponding to 244 bp, while all non *Salmonella* samples gave negative results. All *Salmonella* are reported to carry the *invA* gene which is not carried by any other bacterial species [19]. An exception was observed in the case of one of the isolates previously identified as *Salmonella* by serotyping. Some studies have reported the absence of *invA* gene in *Salmonella* isolates. Rahn *et al*. [25] showed that 2 *Salmonella* serovars they studied did not harbor the *invA* gene, while Fierer and Guiney [14] reported the absence of the gene from *Salmonella* spp. isolated from the environment.

The finding of inv*A* gene in 2 isolates and its absence in another isolate of the same serovar is in line with other findings [14], which reported the gene to be absent in isolates from environmental samples but present in the same serovars isolated from mammals. This could be attributed to the natural deletion of the *invA* gene in the centisome 63 Pathogenicity Island of some environmental isolates [26].

5. Conclusions

In conclusion, this study revealed that the invasion gene (*invA*) was present in *Salmonella* serovars isolated from wastewater, thus highlighting the potential virulent nature of some *Salmonella* serovars associated with wastewater.

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