

Full Length Research Paper

Serotyping and molecular typing of *Salmonella* species isolated from wastewater in Nsukka, Nigeria

Dickson Ihenrochi Dickson, Ike Anthony Chibuogwu* and Ezeonu Ifeoma Maureen

Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

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The objective of this investigation was to isolate and identify *Salmonella* serovars present in wastewater from the University of Nigeria, Nsukka (UNN) wastewater treatment plant and to evaluate the sensitivity and precision of different microbial typing methods (conventional and molecular), in identifying and characterizing *Salmonella* species. A total of 100 suspected *Salmonella* colonies on selective media (*Salmonella-Shigella* agar and MacConkey Agar) were subjected to biochemical testing. A total of 12 biochemically typical *Salmonella* isolates were identified and further characterized. Serotyping analysis further identified 3 (25%) of the isolates as *Salmonella enterica* serovar Limete. *Salmonella* specific (16S) polymerase chain reaction (PCR) assay validated the result obtained by serotyping, although 2 of the isolates could not be serotyped and were identified as rough strains. PCR assay produced positive amplifications of 574 bp of the 16S rRNA gene specific for *Salmonella*, while non-*Salmonella* serovars were negative (100%). Random amplified polymorphic DNA (RAPD-PCR) analysis revealed the genetic relatedness of *Salmonella* serovars isolated from wastewater. Primers 787 and RAPD2 identified 4 RAPD-binding patterns, while primer 1254 did not give any discriminatory pattern. Molecular analyses (16S PCR and RAPD) showed discriminatory power, reproducibility, easy interpretation and performance. It is therefore a promising alternative method for typing *Salmonella* species.

Key words: Wastewater, *Salmonella*, serovars, serotyping, polymerase chain reaction (PCR), 16S rRNA, random amplified polymorphic DNA.

INTRODUCTION

Wastewater may contain millions of bacteria per milliliter including coliforms, Streptococci, Staphylococci, anaerobic spore forming bacilli, *Proteus* and many other types of organisms. Wastewater is also a potential source of many human pathogens including bacteria, viruses and protozoa (Younis et al., 2003). The presence of microbial pathogens in polluted, untreated and treated

water presents a considerable health risk to both humans and animals with far reaching socio-economic implications. *Salmonella* species have been frequently isolated from wastewater and are known to cause severe disease symptoms that range from self-limiting diarrhoea to bacteremia. They are the etiological agents of a wide range of diseases such as salmonellosis and typhoid

*Corresponding author. E-mail: anthonyc.ike@unn.edu.ng. Tel: +234 706 631 7800.

fever and are among the leading causes of gastroenteritis worldwide. There are 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* (CDC, 2005; Bhunia, 2008).

Traditionally, detection and quantification of *Salmonella* species have been largely based on the use of selective culture and standard biochemical methods. This approach requires confirmatory test of all typical and atypical colonies and can be very cumbersome and time consuming (Whyte et al., 2002; Kumar et al., 2009). Since *Salmonella* is closely related to both human and animal health, more rapid and sensitive methods for the identification of this bacterium are required (Schrank et al., 2001). The use of DNA-based typing methods is becoming increasingly important in epidemiological survey and differentiation of *Salmonella* species. Some of these methods include pulsed field gel electrophoresis (PFGE) (Mohand et al., 1999; Kubota et al., 2005), PCR ribotyping (Lagatolla et al., 1996), automated nuclease PCR assay (Hoorfar et al., 2000) and random amplification of polymorphic DNA (RAPD) (Shangkuan and Lin, 1998; Smith et al., 2011). RAPD-PCR does not require any specific knowledge of the target DNA sequence, making it a flexible and powerful tool with general applicability. Furthermore, many oligonucleotide primer sets have been described for the detection of *Salmonella* using the PCR technique (Stone et al., 1994; Guo et al., 2000; Liebana et al., 2001).

In this study, the conventional system for typing *Salmonella* species (biotyping and serotyping) was evaluated and its performance was compared to molecular typing methods (16S and RAPD-PCR analysis).

MATERIALS AND METHODS

Sample collection

A total of 40 wastewater samples were collected from the Imhoff tanks (A and B) and waste stabilization ponds (WSPs) (A and B) of the wastewater treatment plant of the University of Nigeria, Nsukka (UNN), over a period of six months (October 2012 - March 2013). Wastewater samples were collected in sterile bottles and immediately transported to the Microbiology Laboratory, UNN and examined for *Salmonella*.

Isolation and identification of *Salmonella*

Salmonella species were isolated and identified according to the standard methods for the examination of water and wastewater described by ISO (2002) and APHA (2005). Forty milliliters (40 ml) of wastewater was centrifuged at 2000 rpm for 10 min, and then 10 ml of the supernatant was pre-enriched in 100 ml buffered peptone water (BPW) in a 250 ml Erlenmeyer flask and incubated at 37°C for 24 h. Pre-enrichment was followed by selective enrichment in Rappaport Vassiliadis (RV) broth. Subsequently, selectively enriched samples from RV broth were streaked onto *Salmonella-Shigella* Agar (SSA) and MacConkey Agar. Plates were incubated

at 37°C for 24 h. Presumptive *Salmonella* colonies were subjected to a set of biochemical tests for confirmation.

Serotyping

All biochemically typical *Salmonella* isolates were serotyped based on reaction with somatic (O), flagellar (H) and capsular (Vi) antisera (Difco, USA). *Salmonella* O and Vi antigens were identified by slide agglutination test procedure. After the confirmation of the individual *Salmonella* O antisera, cultures were further characterized for H (phase I and II) antisera based on Spicer-Edwards antisera by tube test procedure, whereas, L, EN and I complex antigens were identified separately. The antigenic formulae of *Salmonella* serovars as listed by Popoff and Le Minor (2005) were used to name the serovars. Serotyping was carried out at the WHO Collaborating Centre for Reference and Research on *Salmonella* at Pasteur Institut, Paris, France.

Preparation of genomic DNA

Bacterial DNA was extracted by boiling according to the method described by Medici et al. (2003). A single colony of a pure nutrient agar culture was grown overnight at 37°C in 1 ml Luria Bertani broth. Bacterial cells were pelleted by centrifugation at 13,000 rpm for 5 min in a microcentrifuge (Eppendorf, Germany). The supernatant was discarded and the pellet was re-suspended in 500 µl deionized distilled water. The suspension was boiled for 10 min in a heat block (Techne, Barloworld, UK) then immediately cooled on ice. Extracted DNA was then stored at 4°C until used.

The extracted chromosomal DNA was amplified by an established PCR technique (Sambrook et al., 1989). Bacterial DNA 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 MgCl₂, 0.2 mM of each dNTP, 1U Taq Polymerase), 20 pmol of each primer (Table 1), and ddH₂O to the total volume of 25µl. DNA amplification was done in an Eppendorf vapo protect thermocycler (Hamburg, Germany) following standardized cycling conditions (Table 2).

PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized with a photogel documentation system (Cjinx Science Instrument, USA). For RAPD-PCR, parameters in each reaction were optimized in order to maximize discriminatory power of the reaction. Also, for each PCR assay, a positive control (DNA from *S. Typhi*) and a negative control (sterile distilled water) were included. Appearance of the target band specified for each primer set under specified gel electrophoresis conditions was considered as a positive result.

RESULTS

Identification of *Salmonella* species from wastewater

The result showed that out of 100 *Salmonella*-like colonies on SSA and MacConkey agar, only 65 (65%) were found to be negative for urea utilization. Similarly, of the 65 urea negative isolates subjected to a set of biochemical reactions, 12 (18.4%) isolates were found to be consistent with results expected for *Salmonella* strains (result not shown).

Serotyping of *Salmonella* isolates from wastewater

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Table 1. Primer sequence and reaction parameters.

| Primer pair target | Primer sequence (5'–3') | Annealing temperature (°C) | No. of Cycles | Product size (bp) | References |
|--------------------|---|----------------------------|---------------|-------------------|---------------------|
| 16S rRNA gene | F: TGT TGT GGT TAA TAA CCG CA R: CAC AAA TCC ATC TCT GGA | 56 | 35 | 574 | Lin and Tsen (1996) |
| 787 | AAC GCG CAA C | 36 | 30 | | Smith et al., 2006 |
| RAPD2 | CCC GTC AGC A | 40 | 35 | | Smith et al., 2006 |
| 1254 | CCG CAG CCA A | 36 | 30 | | Smith et al., 2006 |

Table 2. Cycling conditions.

| Step | RAPD primers | | | |
|----------------------|--------------|------------|------------|------------|
| | 16sRNA | 787 | RAPD 2 | 1254 |
| Initial denaturation | 94°C/5 min | 94°C/5 min | 94°C/5 min | 94°C/5 min |
| Final denaturation | 94°C/1 min | 94°C/5 min | 94°C/1 min | 94°C/1 min |
| Annealing | 56°C/1 min | 36°C/1 min | 40°C/1 min | 36°C/1 min |
| Initial extension | 72°C/2 min | 72°C/2 min | 72°C/2 min | 72°C/2 min |
| Final extension | 72°C/10 min | 72°C/5 min | 72°C/7min | 72°C/5 min |
| Hold | 4°C | 4°C | 4°C | 4°C |

revealed that 3/12 (25%) isolates were identified as *Salmonella enterica* belonging to subspecies 1, serogroup B, named *S. enterica* serovar Limete (Table 3). The result further demonstrated that 2 of the isolates could not be serotyped and were identified as rough strains.

Number and percentage of *Salmonella* isolated from wastewater

The result demonstrated that 7.5% of wastewater tested was contaminated with *Salmonella* during the period of study. A total of 3 *Salmonella* isolates were detected and identified, 2 from Imhoff tank A and 1 from WSP B as shown in Table 4.

Salmonella specific PCR (16S rRNA) assay

The specificity and sensitivity of the 16S rRNA PCR assay revealed that 3/12 (25%) of the standard microbiological tests confirmed isolates from wastewater had the desired amplification of 574 bp fragment, whereas no amplicons were observed for non-*Salmonella* serovars (Figure 1).

RAPD-PCR of *Salmonella* isolates from wastewater

The discriminatory power of the RAPD-PCR assay was tested by considering the number of profiles (RAPD binding patterns) generated using a set of 3 primers. The result of the RAPD-PCR

revealed that primers 787 produced 3 similar bands and RAPD 2 produced 1 uniform band for the 3 isolates (Figure 2), making a total of 4 RAPD binding patterns on the basis of genetic relatedness. Primer 1254 did not produce any discriminatory pattern amongst the *Salmonella* isolates.

DISCUSSION

The result of this study revealed a low occurrence (7.5%) of *Salmonella* spp. in wastewater collected from the University of Nigeria, Nsukka wastewater treatment plant (WWTP). This is in consonance with the work done by El Hussein et al. (2012) in Khartoum State, Sudan, who reported an

Table 3. Serotypes of *Salmonella* isolates from wastewater.

| Sample code | Detected O-antigen | Detected H-antigen | Complete antigenic identity (O and H) | Serotype identification (<i>S. enterica</i>) |
|-------------|--------------------|--------------------|---------------------------------------|--|
| A1 | - | - | - | - |
| A5 | O:4, O:12[27] | b:1, 5 | 1, 4, 12, [27]: b.1, 5 | S. Limete |
| A6 | O:4, O:12[27] | b:1, 5 | 1, 4, 12, [27]: b.1, 5 | S. Limete |
| A12 | - | - | - | - |
| A17 | - | - | - | - |
| B18 | - | - | - | - |
| B24 | - | - | - | - |
| B28 | - | - | - | - |
| C1 | - | - | - | - |
| C8 | O:4, O:12[27] | b:1, 5 | 1, 4, 12, [27]: b.1, 5 | S. Limete |
| C28 | - | - | - | - |
| D11 | - | - | - | - |

A = Samples from Imhoff tank A; B = Samples from Imhoff tank B; C = Samples from Waste stabilization pond A; D = Samples from waste stabilization pond B.

Table 4. Number and percentage of *Salmonella* isolated from the wastewater treatment plant, UNN.

| Source | Total samples examined | Total positive samples | Source (%) | Positive samples (%) | Total examined (%) |
|------------------------|------------------------|------------------------|------------|----------------------|--------------------|
| Imhoff Tank A (inlet) | 10 | 2 | 20 | 66.7 | 5 |
| Imhoff Tank B (outlet) | 10 | 0 | 0 | 0 | 0 |
| WSP A | 10 | 1 | 10 | 33.3 | 2.5 |
| WSP B | 10 | 0 | 0 | 0 | 0 |
| Total | 40 | 3 | - | 100 | 7.5 |

WSP = Waste stabilization pond.



Figure 1. *Salmonella*-specific PCR (16S rRNA) of isolates from wastewater. Lane M: 100 bp DNA, Lane 1: Negative control, Lane 2: Positive control (*S. Typhi*), Lane 3-14: samples, Lane 9, 10 and 13 reveals amplification of 574 bp fragments of 16S rRNA gene.

occurrence of 11.09% of *Salmonella* species in wastewater. Conversely, a report by Howard et al. (2004)

showed that municipal wastewater having undergone an activated sludge process continued to bear *Salmonella* at

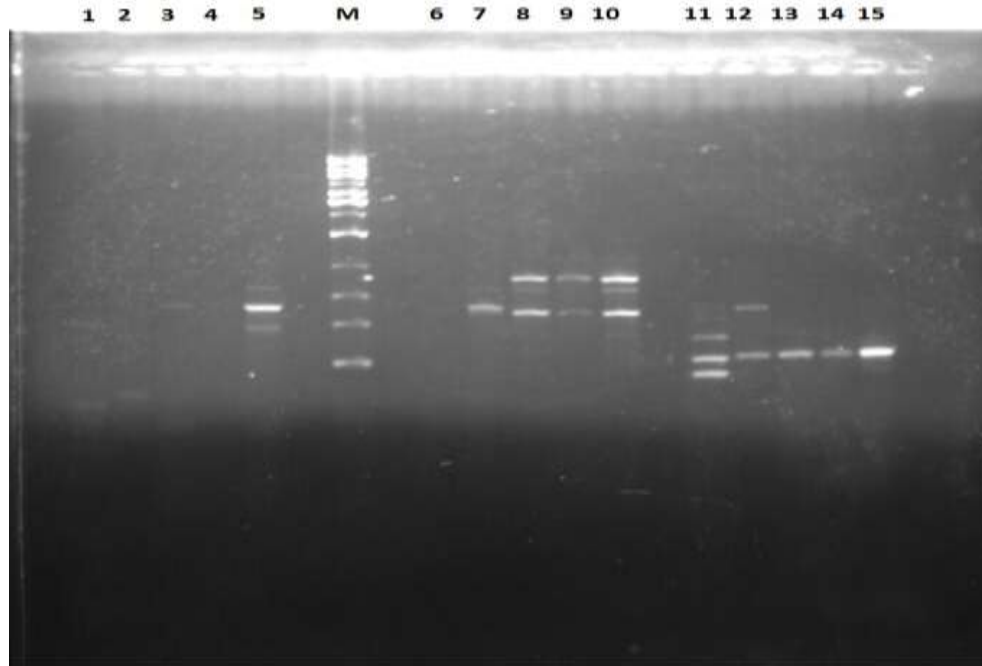


Figure 2. Representative RAPD-PCR of *Salmonella* isolates from wastewater using a set of 3 primers (1254, 787, RAPD 2). Lane M: 1 kb Marker, Lane 3 - 5 (1254), Lane 8 - 10 (787), Lane 13 - 15 (RAPD 2).

high concentration; the treated water yielded a most probable number (MPN) of 45/100 ml. Also, El-Taweel (1994) found that *Salmonella* spp. were detected in raw wastewater samples at oxidation pond in Mit-Mzah treatment plant in Dakahlia governorate, Egypt, in numbers ranging from 10^2 - 10^5 cfu/100 ml.

The result of serotyping was in tandem with the result of the 16S rRNA-PCR assay, although 2 isolates could not be serotyped and were identified as rough strains. Kumar et al. (2009) reported that some of the *Salmonella* serovars isolated from seafood could not be serotyped and were identified as rough strains, lacking O-antigen. This reveals a major limitation of the serotyping technique in subtyping *Salmonella* spp.; as serogrouping is not possible when *Salmonella* isolates lack O-antigen (rough strain) or lack both O and H antigens (Hoorfar et al., 1999). This highlighted the necessity of more discriminatory methods to compliment traditional typing methods. However, since the PCR method could not also identify these as *Salmonella*, it is inferred that this may not have been a limitation in this study.

The specificity and sensitivity of the PCR assay to detect *Salmonella* spp. recovered from wastewater was investigated. The PCR produced positive amplifications of 574 bp of the 16S rRNA gene specific for *Salmonella* serovars, while non-*Salmonella* serovars were negative (100%). The results obtained were similar to those reported by Lin and Tsen (1996) and Chiu and Ou (1996). These investigators reported that 16S PCR

technique using Salm 16S primer was able to identify all the examined *Salmonella* serovars, while all non-*Salmonella* serovars gave negative results.

There was no diversity among the strains analyzed by RAPD-PCR. The discriminatory power of the RAPD-PCR was tested by considering the number of profiles (RAPD binding patterns) generated using a set of 3 primers. The analysis of the RAPD-PCR revealed that primers 787 and RAPD 2 were found useful in typing *Salmonella* isolates and 4 RAPD patterns were observed among the isolates on the basis of shared amplified product showing relatedness of the isolates. Primer 1254 did not produce any discriminatory pattern amongst the *Salmonella* isolates and obviously no typing was possible. This result is in consonance with studies conducted in Lagos, Nigeria by Smith et al. (2011) and Akinyemi et al. (2014). These investigators demonstrated that RAPD-PCR using primer 1254 did not discriminate among *Salmonella* isolates. However, this is in contrast with the report of Quintanes et al. (2004) which recorded highest discriminatory power amongst clinical *Salmonella* isolates using Primers 784 and 1254 in Brazil.

Standardization of PCR mixtures and conditions are very important for reproducibility of RAPD-PCR results. It was found that it was necessary to perform RAPD-PCR in duplicates to obtain valid result. It is important to note that the interpretation of DNA fragment patterns generated by RAPD-PCR requires a good understanding of the occurrence of random genetic events, including

point mutations, insertion and deletions of DNA, which can alter the RAPD fingerprinting pattern (Tenover et al., 1997). For this reason, it was presumed that the differentiating bands in the profiles could be due to one or more genetic events. These findings show that RAPD-PCR yields reliable and reproducible results under precise assay conditions.

The traditional phenotypic typing methods used (biotyping) showed low discriminatory power, while serotyping yielded inconclusive results in few samples. RAPD-PCR analysis showed the potential to provide a discriminatory, reproducible, low cost, easy to perform and interpret method to type *Salmonella* strains. Molecular typing or fingerprinting is an invaluable epidemiological tool that can be used to track the source of infection and to determine the epidemiological link between isolates from different sources. The combination of traditional and molecular typing methods may be the best approach to characterize *Salmonella* isolates.

Conflict of Interests

The authors have not declared any conflict of interests.

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