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ScienceDirect

Procedia Food Science 6 (2016) 334 – 338

Procedia Food Science

International Conference of Sabaragamuwa University of Sri Lanka 2015 (ICSUSL 2015)

Antibiotic resistance and molecular characterization of seafood isolates of nontyphoidal *Salmonella* by PFGE

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Abstract

Emergence of multidrug resistant nontyphoidal *Salmonella* is a major health concern worldwide due to the predominant occurrence of *Salmonella enterica* sub-species *enterica* serovar Typhimurium phage type 104 (DT104) conferring resistance to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline. Apart from antibiotic resistance, the identification and genotypic characterization of pathogens is essential for epidemiological surveillance and outbreak investigations. In this study 39 isolates of *Salmonella* obtained from seafood samples were examined for their susceptibility to various antibiotics and subjected to PFGE analysis using the restriction enzyme *Xba*1. The highest percentage resistance was for erythromycin (100%) followed by nalidixic acid (15.38%), co-trimoxazole (15.38%), chloramphenicol (12.82%), ampicillin (12.82%) and tetracycline (10.25%). Six (15.38%) of the 39 isolates were multidrug resistant. The *Xba*I digested chromosomal DNA generated 7 clusters suggesting the presence of diverse *Salmonella* strains in seafood. The Discriminatory Index for PFGE obtained by *Xba*I restriction enzyme was 0.91. The PFGE has been found highly discriminatory for subtyping *S.* Weltevreden and *S.* Newport. The *Xba*I PFGE was not only discriminatory but could also distinguish multidrug-resistant strains from the sensitive ones as the two groups they belonged to different pulsotypes. The study also demonstrated multiple clones of *S.* Weltevreden, *S.* Newport and *S.* Oslo present in seafood from the south west coast of India. Genetic diversity among the similar seafood sources suggests the presence of different clones of *Salmonella* which further increases the risk of seafood being a potential source of highly pathogenic bacteria like *Salmonella*.

Peer-review under responsibility of International Conference of Sabaragamuwa University of Sri Lanka 2015 (ICSUSL 2015).

Keywords:Salmonella; pulse field gel electrophoresis; antibiotic resistance

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doi:10.1016/j.profoo.2016.02.067

1. Introduction

Emergence of multidrug resistance nontyphoidal *Salmonella* isolates is a major health concern worldwide with the most predominant occurrence of *Salmonella* Typhimurium phage type 104 (DT104) resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline. Agricultural use of antibiotic increases the risk of development of antibiotic resistant zoonotic pathogens such as *Salmonella*[1]. In the recent years emergence of fluoroquinolone resistant *Salmonella* and extended spectrum β-lactam producing strains are of particular concern. Apart from antibiotic resistance the identification and genotypic characterization of pathogens are essential for epidemiological surveillance and outbreak investigations. The molecular subtyping of bacterial strains has become an essential component of outbreak investigations augmenting the identification and trace back of clusters suspected to originate from foods, the environment or nosocomial sources[2]. Epidemiological analysis of *Salmonella* from humans and animals by arbitrarily primed PCR, plasmid profiles and pulse-field gel electrophoresis (PFGE) has been well documented[3]. Pulse-field gel electrophoresis is a technique used for the separation of large DNA molecules by applying to a gel matrix an electric field that periodically changes direction. It is currently considered the gold standard method for subtyping foodborne pathogens[4]. Pulse-field gel electrophoresis (PFGE) has significantly reduced the amount of time required to complete investigations by greatly improving the accuracy of pinpointing sources of foodborne outbreaks due to bacterial pathogens. Providing the evidence of molecular genetic relatedness of two or more strains to the outbreak investigators is the primary epidemiological application of PFGE[5]. It also serves as a basis in the PulseNet national subtyping network for foodborne diseases and has entrenched itself as a powerful tool in the molecular epidemiologic linking of strains during trace back of clusters obtained from different sources. Many studies have been investigated the clonal diversity between or within the different serovars of *Salmonella* by using this typing technique[6,7]. But the PFGE studies on seafood associated nontyphoidal *Salmonella* is limited. Currently PFGE data are considered reliable and a sensitive way to detect differences between closely related strains, so that isolates with indistinguishable PFGE profiles can be classified as epidemiologically linked with a high degree of confidence[4].

2. Methodology

In this study 39 isolates of *Salmonella* obtained from seafood samples were examined for their susceptibility to various antibiotics and subjected to PFGE analysis using the restriction enzyme *Xba*1.Pulse-field gel electrophoresis was performed according to the Standardized Laboratory Protocol for Molecular Subtyping of *Salmonella* by PFGE (Pulse-Net, CDC, Atlanta, USA) (CDC 2012) with slight modifications. Briefly, *Salmonella* cells grown in 5ml LB broth was pelletized and transferred to 2 ml of Cell Suspension Buffer and the concentration of cell suspensions adjusted to 0.8-1.0 OD in a spectrophotometer. 200µl of the cell suspension is then mixed with 20mg/mL of proteinase K and 200µl of 2 % clean cut agarose (Bio Rad, CA, USA) and pipetted into disposable plug moulds (CHEF-DR II, Bio-Rad , CA, USA). After solidification the plugs were removed from the moulds and lysed by using cell lysis buffer. The samples were incubated in a 54 °C shaking water bath for 1.5-3 h with constant vigorous agitation (150-175 rpm). The tubes were removed from the water bath and the lysis buffer was discarded. The plugs were then washed two times with 10-15 ml of sterile ultrapure water (pre-heated to 54 °C) in a 54 °C water bath for 10-15 min with constant agitation. This was followed by four washes with 10-15 ml of sterile 1X TE buffer, pre-heated to 54 °C as described above. After the last wash, 5 ml of sterile 1X TE buffer (room temperature) was added to each tube to serve as storage media for the plugs. The plugs were restricted immediately or stored in 1X TE buffer at 4 °C until use. DNA was then digested with 40 U of restriction enzyme Xba1 (5'-TCTAGA-3') / plug size (Fermentas, U.S.A.) at 37 ºC. The restriction fragments were separated by electrophoresis in 0.5× TBE buffer, for 19 h at 14 ºC in a CHEF Mapper system (Bio-Rad, U.S.A.) using pulsed times of 2.2 to 63.8 s. PFGE data were analyzed using Gel Compar software (BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium)). The relation between the strains was scored by the Dice coefficient of similarity, and strains were clustered by the hierarchical clustering inter-strain similarities based on the unweighted pair group method with arithmetic averages (UPGMA).

3. Results

3.1. Antimicrobial susceptibility

The antimicrobial resistance profile varied among the serovars. Of the 39 seafood isolates of *Salmonella* all were resistant to erythromycin. Two of the 13 *S*. Weltevreden and and 3 of the 9 *S*. Newport isolates were resistant to ampicillin. Two *S*. Weltevreden and 4 *S*. Newport was resistant to both nalidixic acid and co-trimoxazole. The highest rates of resistance were observed for erythromycin (100%), nalidixic acid (15.38%), co-trimoxazole (15.38%), chloramphenicol (12.82%), ampicillin (12.82%) and tetracycline (10.25%). No resistance was observed to ciprofloxacin and kanamycin. Resistance to more than one drug was seen in 4 *S*. Weltevreden and 4 *S*. Newport isolates. Six (15.38%) of the 39 isolates were resistance to 3 or more antimicrobial agents (i.e. multidrug resistant).

3.2. Pulse-field gel electrophoresis analysis

The 39 isolates of *Salmonella* digested using *Xba*I yielded 20 different profiles, which were discriminatory (D=0.91) at 60 % similarity value. A dendrogram based on the similarity value is presented in figure 1. *Xba*I digestion yielded 6 to 14 bands with amplicon size ranging between 48.5 kb to 680 kb. Among the 39 *Salmonella* isolates 26 could be clustered into 7 different groups (X1-X7) showing diversity in profiles, while the other 13 remained unclustered. The unclustered isolates belonged to the serovar*S*. Weltevreden (SW30, SW65 and SW9), *S*. Newport (SN36, SN37, SN35, SN3 and SN70), *S*. Virchow (SV17), *S*. Typhimurium (ST14028), *S*. Oslo (SO9), *S*. Aba (SA74), and *S*. Infantis (SI73). Heterogenity was observed for serovars of *S*. Newport (7 patterns), *S*. Weltevreden (6 patterns) and *S*. Typhimurium (2 patterns). Among the 13 isolates of *S*. Weltevreden 6 isolates (46.15 %) belonged to the PFGE cluster X1, 2 (15.38 %) each to X2 and X3, while the remaining three (23.07 %) showed individual distinct patterns. The 9 *S*. Newport strains were observed to group into two clusters designated X6 (4 strains) and X7 (4 strains) while the other 5 remained unclustered at 60 % similarity. Ten among the 11 *S*. Oslo strains formed a single group (X4) which was further sub-clustered in to two, where one subgroup clustered *S*. Oslo (2 strains) isolated from oysters and another group form a mixture of *S*. Oslo isolated from squids and oysters. *S*. Typhimurium grouped in a cluster designated as X5. The single strain of *S*. Oslo (SO9) from oyster clustered with S. Newport (SN3) at 53 % similarity. The single strains of *S*. Aba, *S*. Virchow and *S*. Infantis clustered with *S*. Typhimurium, *S*. Weltevreden and *S*. Newport at a similarity of 55 %, 57 % and 35 % respectively. All the serovars typed in this study were isolated from a particular seafood source. *S*. Weltevreden isolates grouped in cluster X1 were isolated from fish and shrimp; whereas cluster X2 included serovars isolated from fish. Similarly, the clusters X6 and X7 of *S*. Newport were generated from the serovars isolated from clam. The clusters X3, X4 and X5 of *S*. Weltevreden, *S*. Oslo and *S*. Typhimurium were generated from the serovars isolated from mixed animal sources of squid/ oyster, oyster/shrimp and fish/clam respectively.

4. Discussion

4.1. PFGE analysis

The ability of *Xba*I to successfully discriminate between 39 strains analyzed differed. The DI value for PFGE obtained by using *Xba*I was 0.91 (> 0.90) which is the acceptable confidence value for understanding the degree of discrimination[8]. *Xba*I PFGE analysis with 7 different patterns could differentiate the seafood isolates indicating the presence of diverse *Salmonella* serotypes in this region. High genetic diversity and limited genetic similarity were observed for *Xba*I restriction digested *S*. Newport serovars. The PFGE method has been proven to be highly discriminating in this study for subtyping *S*. Weltevreden and *S*. Newport which is in agreement with the earlier studies, showing high discrimination for subtyping of *S*. Typhi and *S*. Typhimurium. This could be evident by the observation of six *Xba*I digested DNA banding patterns for 13 *S*. Weltevreden strains and 7 *Xba*I digested DNA banding patterns for 9 *S*. Newport strains. The cluster X3 and X4 of *Xba*I PFGE pattern (Fig. 1) contains strains which are almost genetically similar to one another. Thus, even though these isolates are from different seafood sources (shrimp/oyster for X3 and squid/oyster for X4); the strains could be clonally related. Similarly, the serovar*S*. Oslo seemed to be fairly homogenous with strains originating from squid and oyster of cluster X4 being genetically similar, though they were isolated from different sources. The *Xba*I PFGE analysis used in this study could also be able to distinguish multidrug-resistant strains (SN36 and SW9) from the sensitive strains (SN71 and SW30) as they shared

different pulsotypes. Genetic diversity among the similar seafood sources suggests the presence of different clones of *Salmonella* which further, increases the risk of seafood being a potential source of highly pathogenic bacteria like *Salmonella*. Regardless, these data confirm the observation that multiple clones of *S*. Weltevreden, *S*. Newport and *S*. Oslo can be isolated throughout the south west coast of India.

In conclusion, PFGE is the widely used molecular tool to determine the diversity among *Salmonella* isolates. It provides a suitable tool for the epidemiological typing of different *Salmonella* serovars isolated from seafood. Subtyping of *Salmonella* by PFGE can also be extremely useful when assessing contamination and dissemination problems in different environments apart from epidemiological investigations. Molecular fingerprinting by PFGE in this study showed relatively high diversity among the *Salmonella* serovars isolated from seafood, suggesting contamination from diverse sources.

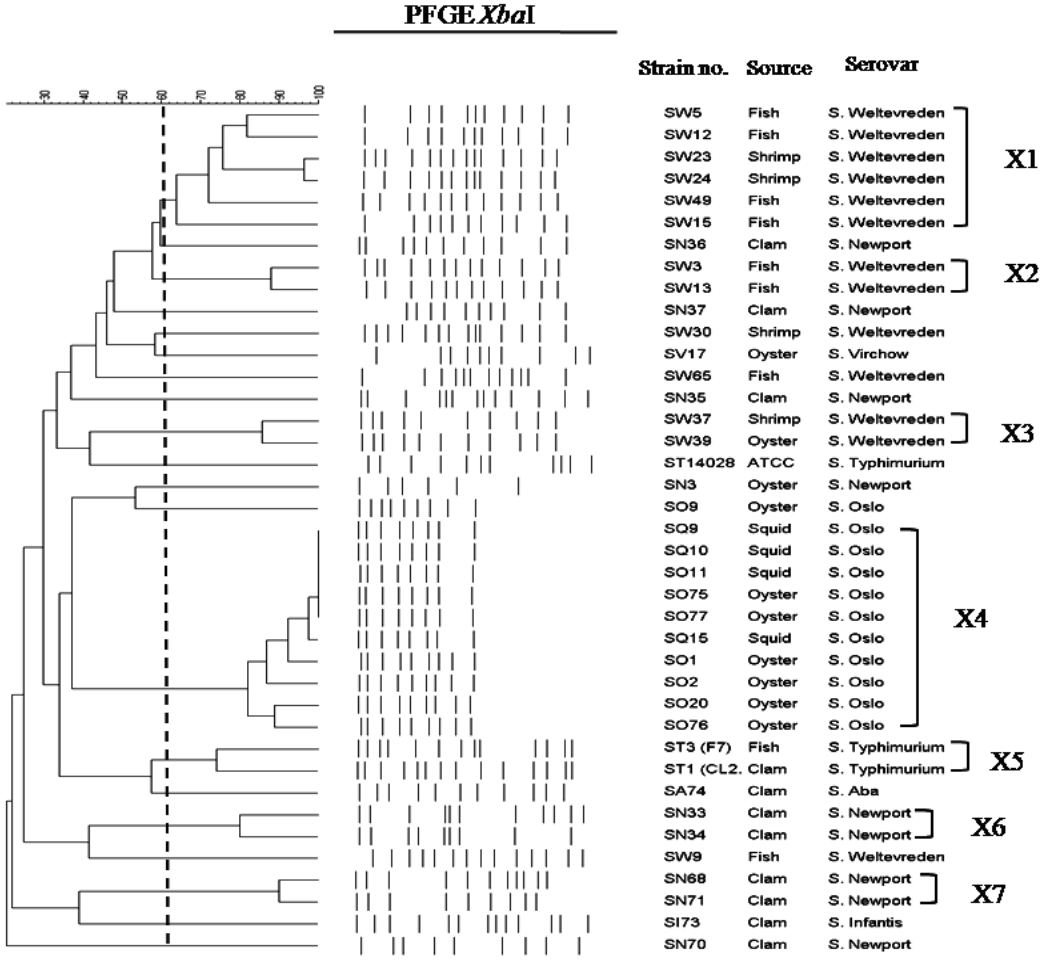

Fig. 1.Dendrogram showing percentage similarity between type-able seafood associated *Salmonella* generated from *Xba*I digested pulse-field finger printing. X1-X7 represent the different PFGE profiles resolved at 60% similarity. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) method.

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Acknowledgements

The financial support provided by the Indian Council of Medical Research through the project on Antimicrobial resistance (AMR/37/2011-ECD –I) is gratefully acknowledged.

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