OPEN ACCESS Plant Protection, 08 (04) 2024. 581-588 DOI:10.33804/pp.008.04.5251

Available Online at EScience Press
Plant Protection
ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

Research Article

PHYLOGENETIC ANALYSIS OF *SALMONELLA ENTERICA* ISOLATED FROM IMPORTED AND LOCAL POTATO SEED TUBERS

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ARTICLE INFO

Article history
Received: 15th June, 2024
Revised: 10th October, 2024
Accepted: 16th November, 2024

Keywords
invA gene
RAPD-PCR
Salmonella enterica
Food safety
Rapid detection
Lineage diversity

ABSTRACT

Human pathogens on plants are an emerging global health concern. *Salmonella enterica* exhibits a broad range of host associations, including plants, animals, and humans. Therefore, the rapid characterization of *Salmonella* on potatoes is essential for ensuring food safety, protecting crops, and preventing transmission to humans. The present study investigates the phylogenetic relationships of potato-borne *S. enterica* with clinical lineages. Among sixty-seven *S. enterica* isolates collected from local and imported potato seed varieties, 63 (94.02%) were confirmed to carry the *invA* gene, a specific marker for *Salmonella*. Random Amplified Polymorphic DNA (RAPD) analysis grouped these isolates into three distinct clusters, with *Salmonella* strains 7S-Moz-L and 1S-Kar-I showing genetic similarity to the clinical strain *S. typhi* 5S-CL2 (MZ708960). The *invA* protein is identified as a virulence factor relevant to human infections and may play a role in tuber colonization. Phylogenetic analysis of *invA* sequences revealed homology between *Salmonella* strain 9SF-Ast-L (MW319054), isolated from the Asterix potato variety, and the clinical strain *S. typhi* 5S-CL2. Moreover, *Salmonella* strain 1S-Kar-I [MW319050], isolated from the imported Karuda variety, showed an evolutionary relationship with *Salmonella* strains 7S-Moz-L (MW319052) and 8S-Qas-L (MW319053), both from local potato varieties. Overall, the study underscores the importance of identifying *S. enterica* in potato seeds prior to planting or transport to prevent the spread of quarantine pathogens and ensure public health safety.

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INTRODUCTION

Contamination of fruits and vegetables with human pathogens has become increasingly common. Human pathogens on plants (HPOPs) can adhere to, penetrate, and colonize plant tissues, posing a risk to both farmers and consumers (Wang et al., 2020). The presence of HPOPs underscores the potential role of plants in transmitting these pathogens to humans and highlights

the importance of source tracking for vectors and pathogens that move between agricultural environments and human populations (Fletcher et al., 2013; 2017). Although plant, animal, and human pathogens follow distinct evolutionary paths, their transmission is not strictly limited by these distinctions; pathogens can be spread across different kingdoms of life through horizontal [soil] and vertical [seed] transmission pathways. Both plants and the rhizosphere may serve as reservoirs for human pathogens, with instances of cross-transmission, though relatively rare, documented in the literature (Kim et al., 2020).

Several bacterial species, including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Erwinia* spp., *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogenes*, are recognized as cross-kingdom pathogens (Vidaver et al., 2006; Mendes et al., 2013). Among these, *Salmonella* species are a significant cause of foodborne gastroenteritis [salmonellosis], responsible for approximately 93.8 million foodborne illnesses and 155,000 deaths annually (Heredia and García, 2018). Recent increases in food poisoning cases have been linked to consuming contaminated raw fruits and vegetables (Wiedemann et al., 2015).

The mechanisms by which *Salmonella* adapts to diverse hosts (animals or plants) are not yet fully understood. However, various pathogenicity factors facilitate *Salmonella*'s ability to colonize and internalize within both plant and animal hosts. The *invA* protein, an inner-membrane component of the *Salmonella* type III secretion system (T3SS), encoded by pathogenicity island 1 (SPI-1) on either chromosomal or plasmid DNA, is a prominent pathogenicity factor found across strains infecting plants, animals, and humans (He et al., 2004; Worrall et al., 2010). In plant-associated strains, T3SS suppresses plant immune responses and enhances pathogen adherence (Schikora et al., 2012; Zarkani and Schikora, 2021).

Crops grown in soil are particularly susceptible to human pathogens due to contaminated compost and irrigation water. In infected rhizosphere environments, seeds and roots can facilitate the internalization of *Salmonella* species, allowing the bacteria to move through the vascular system of plants to edible parts. This process can make crops a prevalent source of foodborne pathogen dissemination (Fletcher et al., 2013; Gu et al., 2013; Mendes et al., 2013; Wiedemann et al., 2015; Jechalke et al., 2019; Luu et al., 2020).

The present study was designed to trace the sources of *S. enterica* associated with both locally grown and imported potato tubers, aiming to distinguish between vegetable-borne and clinical lineages and to analyze their phylogenetic relationships by combining *invA* gene sequencing with RAPD-PCR techniques. The *invA* gene serves as the molecular target for *Salmonella*-specific detection methods recommended by the U.S. Food and Drug Administration's Bacteriological Analytical Manual. Furthermore, *invA* shows evolutionary diversity across *Salmonella* serovars, allowing its sequencing to detect lineages from various sample types (Buehler et al., 2019; Kadry et al., 2019).

MATERIALS AND METHODS

Collection of sample

Three imported varieties of potato seed tubers viz. Karuda, Asterix, and Ultra were obtained from the Department of Plant Protection, Malir Halt, Karachi, Pakistan. Moreover, four varieties, including Mozika, Asterix, Qasur, and Lady Rosetta, were sourced from the local market. Ten tubers from each variety were randomly selected for the isolation *Salmonella* species.

To assess phylogeny and lineage diversity among potato-borne *Salmonella*, reference strains *S. typhi* MDR 5S-CL2, *S. typhi* XDR ST-CL3, and *S. enterica typhi* ATCC 6539 were included in the study.

Isolation of *Salmonella* species

Salmonella species were isolated through a pre-enrichment process. Briefly, surface-sterilized potato seed tubers were cut into 1 cm strips and inoculated into trypticase soy broth (9 ml). After overnight incubation at 37°C, loopful enriched cultures were streaked onto bismuth sulfite agar (BSA, Oxoid, UK) and incubated for 36 h at 35°C. Black, shiny colonies isolated from the BSA plates were subcultured onto nutrient agar plates for further analysis. *Salmonella* species were primarily identified using biochemical and serological tests according to Bergey (1994). Selected strains were further subjected to molecular identification and phylogenetic analysis, following the methodology of Garrity et al. (2005).

Molecular identification of *Salmonella* species

Genomic DNA extraction and *Inv* gene amplification

Genomic DNA was extracted using the CTAB method and subsequently used as a template for identifying *Salmonella* isolates associated with potatoes. Briefly, *Salmonella* species were detected by targeting the

invasion protein-specific primers Inv-SW-F (5'-TCGTGACTCGCGTAAATGGCGATA-3') and Inv-SW-R (5'-GCAGGCGCACGCCATAATCAATAA-3'), following the protocol of Aye et al. (2019). PCR amplification was carried out on a Bio-Rad T100 thermal cycler, using 2× DreamTaq Ready Mix [Thermo Scientific, USA]. The resulting ~420 bp amplicons were resolved via 1.5% agarose gel electrophoresis to confirm the purity of the PCR products (Hasan et al., 2018).

RAPD PCR and cluster analyses

RAPD-PCR amplifies random regions within a genome of an organism, producing distinct banding patterns that are valuable for strain differentiation and assessing lineage diversity (Albufera et al., 2009; Hasan et al., 2023). RAPD fingerprints were generated using the RAPD-M13 primer (GAGGGTGGCGGTTCT) and 2× Dream Taq Ready Mix (Thermo Scientific, USA), following the protocol outlined by Madalena et al., (2010). The resulting RAPD fingerprints were separated on 1.7% agarose gels containing ethidium bromide, run at 70V for 1.5 h, and then analyzed using distance matrices for clustering. The phylogenetic relationship between potato-borne and clinical isolates was determined through UPGMA and Pearson correlation methods using GelClust software (Khakabimamaghani et al., 2013; Rehman et al., 2021).

Sequencing, speciation, and lineage characterization

InvA gene amplicons were cleaned and concentrated using the DNA clean and concentrator kit (Murphy Ave., Irvine, USA) according to the manufacturer's instructions before performing di-deoxy chain termination sequencing PCR. Sequencing was conducted on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific). Species identification was confirmed through a BLAST search (NCBI), and the sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI), USA, to obtain accession numbers. Phylogenetic analysis was carried out following multiple sequence alignments using MUSCLE, and cluster differentiation was performed through UPGMA in MEGA-X software (Edgar, 2004; Kumar et al., 2016). For lineage characterization and cluster differentiation, eight sequences from local and imported potato seed tubers were compared with *S. typhi* CL2 and randomly selected sequences from the NCBI GenBank database.

RESULTS AND DISCUSSION

Rapid detection and characterization of foodborne *Salmonella* are of utmost importance, as potato seed tubers can serve as a reservoir for *Salmonella* species used in crop production. This study highlights the prevalence of *S. enterica* in both local and imported potato varieties (Table 1). A total of 67 *Salmonella* isolates were recovered from potato seed tubers sourced locally and from imports. Nearly 80-100% of local varieties, including Asterix, Qasur, and Mozika, were contaminated with *S. enterica*, while 40% of Lady Rosetta tubers harbored *Salmonella* species (Table 1). Similarly, imported seed tubers, including Asterix (100%), followed by Karuda and Ultra, were infested with *S. enterica* (Table 1).

Table 1. Occurrence of *Salmonella enterica* in potato seed tubers.

Varieties	Names	*Salmonella sp.*	Code
Local Variety	Asterix	100%	1S & 2S
Local Variety	Lady Rosetta	40%	3S
Local Variety	Qasur	80%	4S
Local Variety	Mozika	90%	5S
Imported Variety	Asterix	100%	6S
Imported Variety	Ultra	100%	7S & 8S
Imported Variety	Karuda	90%	9S

The presence of *Salmonella* species in seed tubers was detected using a *Salmonella*-specific *invA* gene probe. As shown in Figure 1a, representative amplicons of the *invA* gene were detected in 63 (94.02%) out of 67 isolates. Moreover, variation in the *invA* gene product [420-600 bp] revealed genetic heterogeneity, potentially caused by single nucleotide polymorphisms (SNPs) or differences in priming sites as shown in Figure 1a.

DNA fingerprints, shown in Figure 1b, highlight two regions of differential RAPD elements (RDRE). The heterogeneic RDRE regions of 100-500 bp and 750-10,000 bp distinguished genetically different *S. enterica*

isolates from local and imported seed tubers as well as clinical sources. The dendrogram in Figure 1c illustrates the phylogenetic relationship of *S. enterica* isolates, created using GelClust software with the UPGMA-Pearson correlation method. The isolates are grouped into three clusters (A-C), each containing three strains, with node evaluation rates ranging from 0.01 to 0.108. Sub-clades 0.336-0.453 exhibit high cluster reliability and genetic similarities within the group.

Clusters A and B, which include isolates from seed tubers, show an environmental association, while *Salmonella* 7S-Moz-L and *Salmonella* 1S-Kar-I in cluster C exhibit genetic relatedness to *Salmonella* 5S-CL2, a clinical strain in Figure 1b. Thus, RAPD-PCR can be a rapid, reliable, and accurate method for differentiating environmental and clinical *Salmonella* strains in epidemiological surveys, especially when compared to traditional biochemical-based identification methods (Hasan et al., 2018).

Furthermore, sequences of the *invA* gene obtained from *Salmonella* 1S-Kar-I, *Salmonella* 2S-Ast-I, and *Salmonella* 3SR-Ult-I (from imported seed tubers: Karuda, Asterix, and Ultra), as well as five isolates (*Salmonella* 6S-Lro-L-NT1405, *Salmonella* 4S-Moz-L-NT1450, *Salmonella* 7S-Moz-L, *Salmonella* 8S-Qas-L, and *Salmonella* 9SF-Ast-L) from local varieties (Lady Rosetta, Qasur, Mozika, and Asterix) were used to infer phylogenetic relationships and lineage diversity.

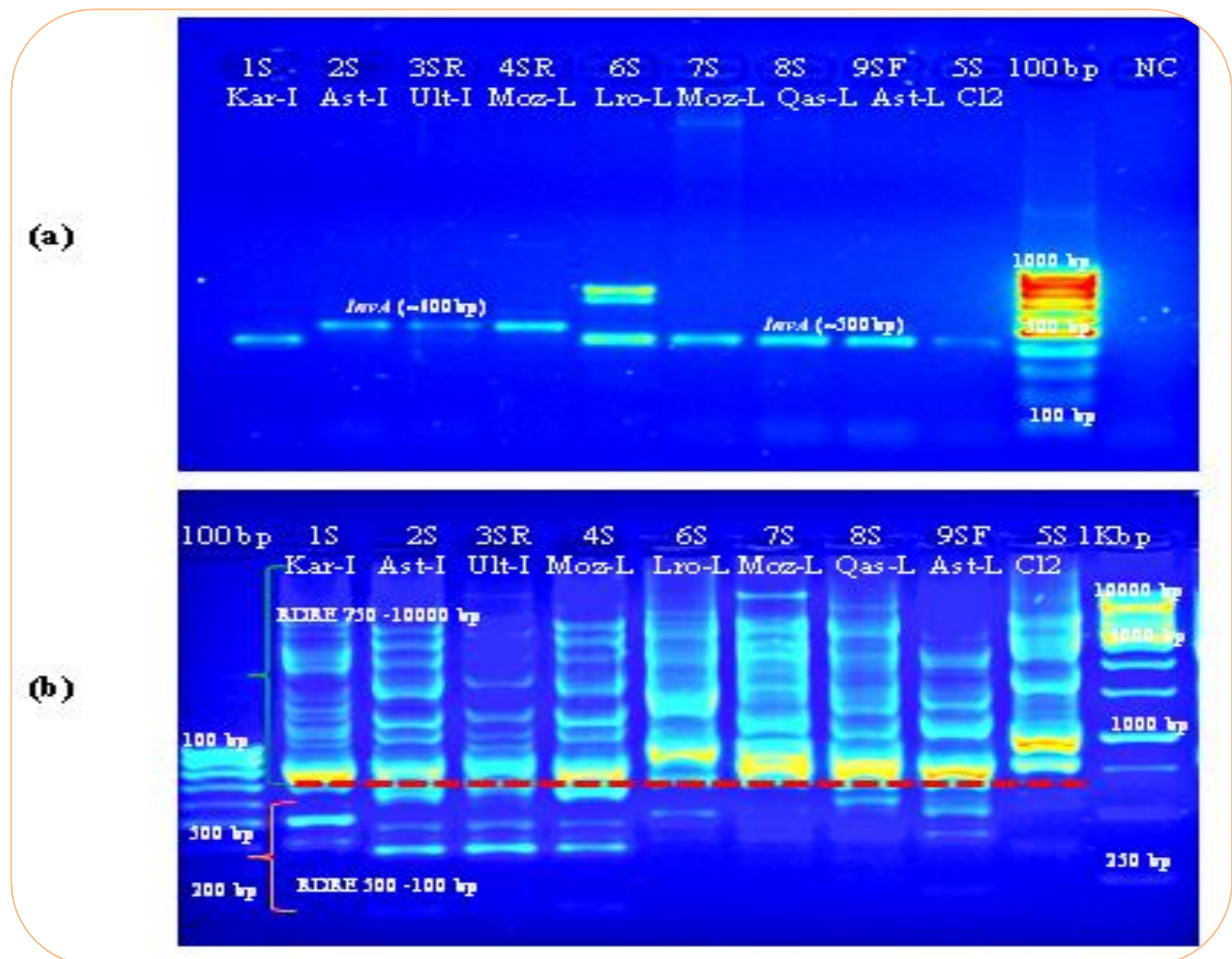

Figure 1. Agarose gel electrophoresis of *invA* gene PCR amplicons used as a molecular barcode for the identification of *S. enterica* isolated from local and imported potato seed tubers. a: shows genetic variation in *invA* amplicons (420-600 bp); b: RAPD-PCR fingerprints of *Salmonella* isolates exhibit intra-strain variations between *S. enterica* isolated from local and imported potato seed tubers.

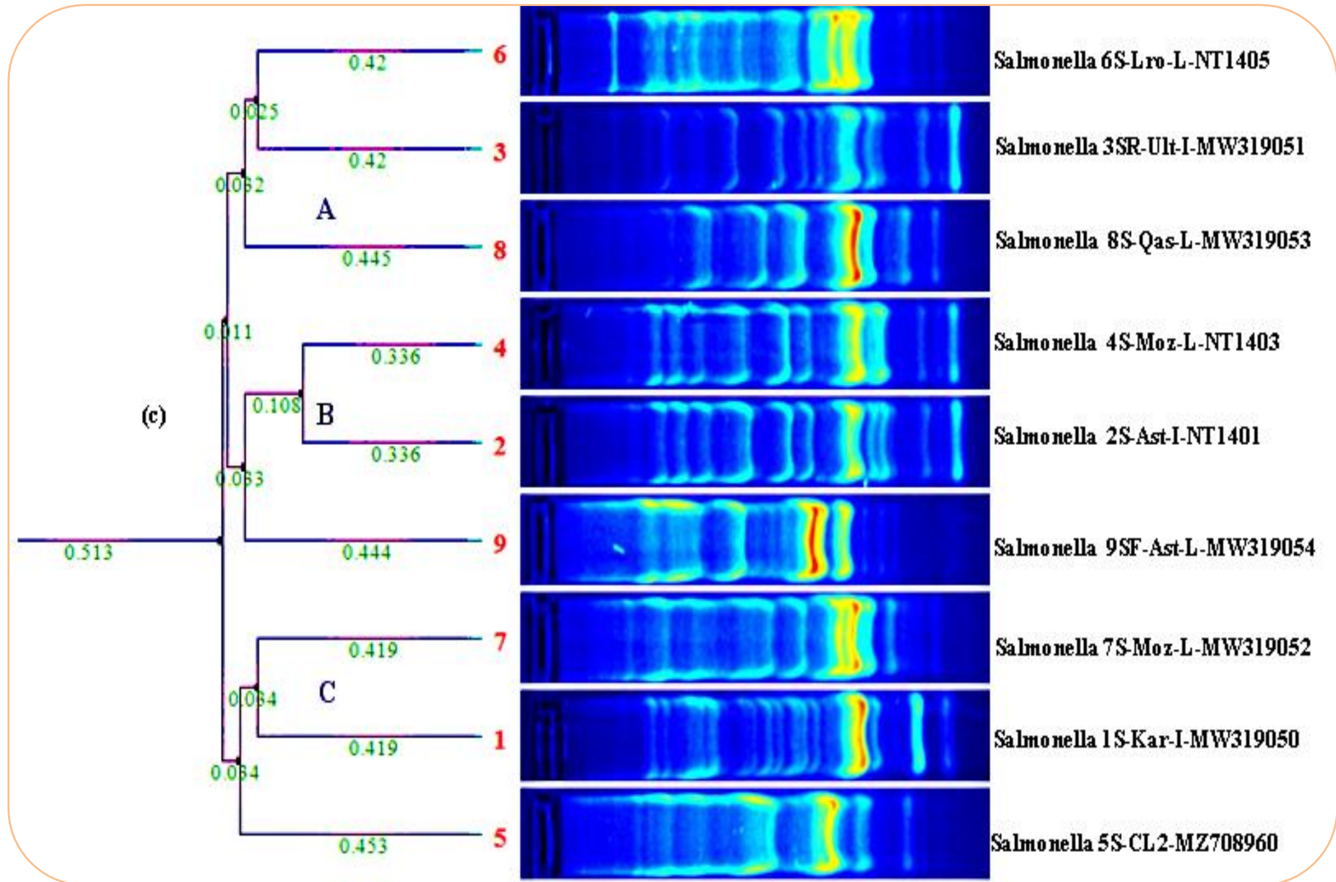

Figure 1c. Dendrogram based on the UPGMA-Pearson correlation method depicting the phylogenetic relationship between potato-borne *S. enterica* and a clinical isolate.

The *invA* gene sequences of potato-borne *Salmonella* isolates 1S-Kar-I, 3SR-Ult-I, 7S-Moz-L, 8S-Qas-L, 9SF-Ast-L, and a clinical isolate *S. enterica typhi* 5S-CL2 were deposited in GenBank under the accession numbers MW319055, MW319051, MW319052, MW319053, MW319054, and MZ708960, respectively.

The evolutionary history, intra-species diversity, and cluster differentiation of the *invA* gene were inferred by comparing eight selected isolates from local and imported seed tubers with clinical and food-borne *S. enterica* strains obtained from NCBI GenBank. The phylogram in Figure 2 clustered the *Salmonella* isolates into two clades, A and B, with 95% sequence coverage. Clade A mainly consisted of clinical *Salmonella* strains and exhibited a 99% cluster cut-off. Moreover, *Salmonella* 9SF (MW319054) from Asterix seed tubers and *S. enterica* CP040380.1 from pistachio demonstrated genetic relatedness to *S. enterica typhi* 5S-CL2 as shown in Figure 2. Interestingly, clade B [95% cluster cut-off] was distinguished from clade A by a 4% cluster divergence, primarily due to differences in the amino acid sequence of the *invA* protein. Seven isolates were clustered within clade B, including *Salmonella* 6S (NT1405), *Salmonella* 1S-Kar-I (MW319050), *Salmonella* 7S-Moz-L (MW319052), and *Salmonella* 8S-Qas-L (MW319053), which exhibited associations with one another (cluster cut-off: 95-67%). Meanwhile, *S. enterica* NT1405 showed intra-species differences, as indicated by the branch length (0.016). Furthermore, *S. enterica* 2S (NT1401) and *S. enterica* 3SR (MW319051), isolated from imported tubers (Asterix and Ultra), displayed genetic relatedness in Figure 2. The *S. enterica* NT1403 strain was the most genetically divergent, with evolutionary distances of 0.110.

The genetic diversity within the *invA* gene sequences highlights its significance for phylogenetic clustering and lineage association when isolated from food and clinical sources as shown in Figure 2. Moreover, the *invA* gene is a common virulence determinant, capable of injecting effector proteins directly into various host cells, including those of plants, animals, and humans.

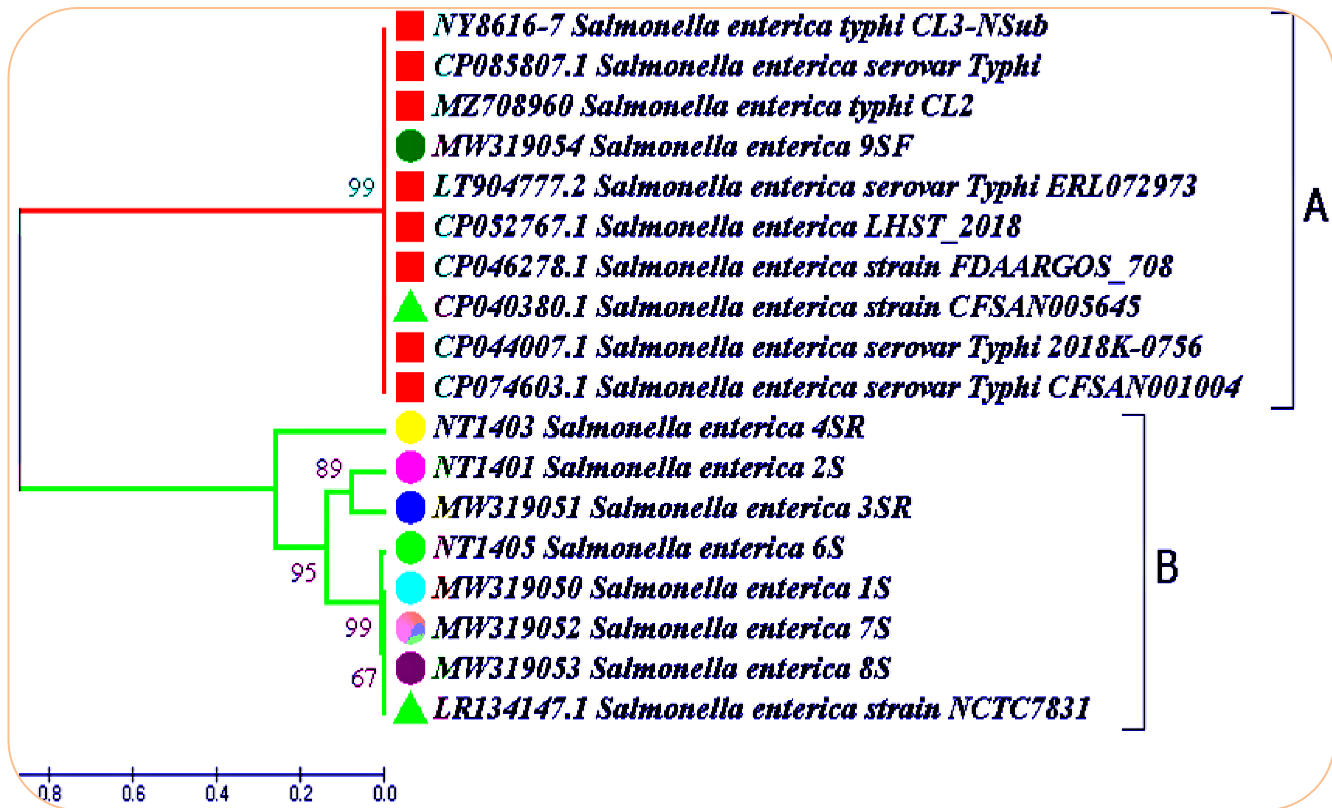

Figure 2. Phylogenetic relationships among *Salmonella* isolates from potato seed tubers and their genetic relatedness to clinical strains were inferred by calculating the number of amino acid substitutions per site in the *invA* protein sequences using the Poisson correction method. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis included 18 amino acid sequences, with positions having less than 95% site coverage excluded. The optimal tree, with a sum of branch lengths equal to 2.23127213, is shown. The percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated next to the branches, reflecting intra-species diversity and cluster differentiation among eight selected isolates from both local and imported seed tubers.

Numerous food products are being rejected in international markets due to the presence of human pathogenic bacteria, particularly *Salmonella* species (Pigłowski, 2019). Potatoes are consumed in various forms, including cooked, frozen, dried, ready-to-eat, and minimally processed items, as part of daily life. The increasing market demand for both new and existing potato products has heightened the need to ensure microbial safety. Planting healthy potato seeds is a key factor in maximizing potato production for safe consumption and processing (Malek et al., 2013). Therefore, measures to prevent the spread of foodborne pathogens during the pre-harvest stage [at the potato seed tuber sowing stage] are essential for producing healthy crops for human consumption. Source tracking and lineage characterization of *Salmonella* species originating from plants are crucial for safeguarding human health. In this study, the presence of *Salmonella* in potato samples may be attributed to factors such as the use of contaminated irrigation water, manure application as fertilizer, post-harvest processing, storage, and shipping (Malek et al., 2013; Zarkani and Schikora, 2021; Mkangara, 2023). Even though the occurrence of enteric bacteria in commercial potato seeds may be unavoidable, characterizing the lineages of *S. enterica* to track pathogenic strains may provide valuable understandings of their sources, plant-microbe interactions, environmental impacts, and food processing. Therefore, molecular methods such as RAPD-PCR, *invA* PCR, sequencing, and phylogenetic analysis can aid in developing evidence-based policies and procedures to minimize the spread of *Salmonella* and improve safety standards for potato seed tubers free from *S. enterica*.

CONCLUSION

Pre- and post-harvest crop protection against enteric pathogens, such as *Salmonella*, requires constant and rapid surveillance strategies that can track and identify strains using molecular barcodes. Combining RAPD-PCR with the sequencing of *Salmonella*-specific molecular barcode (*invA*), can help develop a surveillance system for the rapid identification and characterization of the source and lineage of these pathogens. This approach may prevent the transfer of *Salmonella* from seeds to humans. Moreover, accurate and timely identification of foodborne *Salmonella* can help restrict unsafe imports and exports, thereby preventing the entry of infected potato tubers into the country and the food chain.

ACKNOWLEDGMENTS

We are thankful to the Department of Plant Protection, Government of Pakistan, Malir Halt, Karachi, Pakistan for providing samples of imported potato seed tubers.

AUTHORS' CONTRIBUTIONS

RZ and SN collected potato seed tubers, isolated and identified *Salmonella*, and compiled the data; RZ, SN, and FN performed the data analysis, interpreted the results, and wrote the article; NM, SFHN, and MF provided clinical strains and ATCC cultures and assisted in data analysis; SS critically revised the manuscript; KAH conceived the idea, designed the studies, performed the experiments, analysed the data, interpreted the results, wrote the manuscript, made critical revisions, and gave final approval of the draft; All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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