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Molecular Identification and Characterization of *Salmonella Sp.* In Raw and Heat-Treated Milks from Local Market

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Abstract

Milk is an excellent nutritious food that is characterized by a short shelf life and requires careful handling. Therefore, milk is rapidly perishable as it is a suitable medium for the pathogenic bacterial growth especially; causing milk spoilage and diseases in consumers. Nine Salmonella isolates recovered from 4 (8%) and 5 (10%) samples of Raw cow milk and Raw buffalo milk, respectively while no isolates of *salmonella sp.* were detected from heat-treated milk. Moreover, the antimicrobial susceptibility testing showed that nine isolates were resistant to Amoxicillin/clavulanic acid (AMC 30) 20/10µg, Erythromycin (E15) 15µg and Cephalexin (CL30) 30µg whilst they are all sensitive to Chloramphenicol (C30) 30µg. Additionally, our isolates revealed that they were posed 16S rRNA gene and by using amplified ribosomal DNA restriction analyses (ARDRA) technique the resulting restriction fragment patterns, showing that all isolates following the same genus and species. Importantly, two isolates were sequenced and submitted in NCBI under accession numbers OR048345 and OR102504. The virulence gene profile of the nine isolates revealed that they are poses the six virulence genes (*invA*, *orgA*, *sifA*, *spvA*, *iroN* and *spiA*) were detected significantly in all nine isolates. A mimic experiment was done to estimate the sensitivity of the real time-PCR to detect *salmonella sp.* in contaminated milk samples. Our results indicate that insufficient hygienic measures adopted during handling. Therefore, the strict hygienic approaches are recommended during milking and processing. The current study aimed to determine the prevalence of *Salmonella sp.* in raw and heat-treated milk, characterize its virulence-associated genes, and assess its antimicrobial profile.

Keywords: *Salmonella sp.*; Virulence- associated genes (*invA*, *orgA*, *sifA*, *spvA*, *iroN* and *spiA*); ARDRA, PCR, multiplex PCR, RT-PCR.

Introduction

Milk is the main contributed food product with calcium, mostly of children and women, but also of adolescents and the elderly. Thus, it is an essential source of offspring nourishment, differ in its composition and properties during the production and processing procedures significantly retaining its nutritional value (Dominguez-Salas et al., 2019). Further, milk is almost sterile when secreted from a healthy udder (Cao et al., 2021; Chege et al., 2016) and milk has antimicrobial properties due to contain the lactoperoxidase, lysozyme and lactoferrin proteins and further possibly N-acetyl-ß-D- glucosaminidase, which may be involved in protecting against mastitis, bacterial growth post-harvest, and the consumer of the milk product (Owusu- Kwarteng et al., 2020; Embleton et al., 2013). Consequently, it prevent significant rises in the number of bacteria at ambient temperatures for the first 3 to 4 hours after milking (Owusu- Kwarteng et al., 2020; Murata et al., 2013).

Milk can be considered as a source of disease transmission, because it is an ideal medium for microbial growth due to its high nutrient content, high moisture and the near neutral pH (6.4–6.8) (Ritota et al., 2017). Moreover, the presence of foodborne pathogens in milk is influenced by various factors. These factors include the farm size, the number of farm animals (Rahman et al., 2018) and management practices of farm. Further, the evaluation and detection of variation in sampling, methodologies used, samples types, seasons and the geographical location play critical role in milk contamination by foodborne pathogenesis (Deddefo et al., 2023; Velázquez-Ordoñez et al., 2019). It has been reported that the direct contact with foodborne pathogens in milk is closed to the dairy farm environment such as (soil, the water supply quality, housing conditions, teat canal,

surface of teat skin, animal's feed, faeces and equipment hygiene) (Cancino- Padilla et al., 2017) soil, (Chege and Ndungu, 2016).

The most frequently isolated bacteria from milk contains *Salmonella sp.*, Staphylococcus aureus, Escherichia coli O157:H7, Listeria monocytogenes and Campylobacter. Among these pathogens, Salmonella sp. which considered as main microbiological hazards associated with raw milk consumption (Gebeyehu et al., 2022; Idland et al., 2022; Berhe et al., 2020).

Salmonella is a zoonotic pathogen that affects different animal species and causes salmonellosis, which is the most common foodborne disease reported (Dawood Saleem et al., 2022; Cremonesi et al., 2020). Salmonellosis affects humans causing gastrointestinal disease (Shalal et al., 2023) and may cause death in children (Castañeda- Salazar et al., 2021; Popa and Papa, 2021).

Importantly, *Salmonella sp.* virulence tactics to interact with the host defense mechanisms, there were 17 genes are associated with Salmonella invasion, iron transport, intra macrophage survival, toxin production, fimbrial expression and apoptosis. These genes were found on a virulence plasmid (pSLT) or in the chromosome, the majority of the genes encoding the most significant virulence components are found within highly conserved Salmonella pathogenicity islands (SPIs). Currently, five SPIs (SPI-1 to SPI-5) and other virulence factors like the spv operon carried by the pSLT plasmid, various adhesin types and flagella. Further, invA gene is a specific biomarker for Salmonella spp. as containing unique sequences to the genus Salmonella. In addition, SifA gene, located in chromosome, decrease of kinesin accumulation in the SCV, modulation of vesicular trafficking, SCV perinuclear migration, SCV membrane integrity and SipA, located in SPI-1, Actin Stabilization and localization of actin filaments during invasion, stabilization of VAP, correct localization of SifA and PipB2, SCV perinuclear migration and morphology (Shalal et al., 2023; Maaroof and Dhayea, 2023; Pławińska-Czarnak et al., 2023; Yuliangsih et al., 2019).

The important conventional techniques used for detection of microbes in milk are generally laboratory-based, consumed time, and labor intensive (Dawood Saleem et al., 2022; Moghadam et al., 2022) and unable to analyze new organisms, thus milk and dairy products tests need rapid detection techniques. While, the rapid detection techniques are specific, efficient, sensitive, less time consuming and more reliable than conventional techniques. For examples, the polymerase chain reaction (PCR), multiplex- PCR, real-time PCR and DNA microarrays are classified as rapid detection techniques. Further, biosensor based technology and immunological based methods can be used as rapid detection techniques (Hameed et al., 2018).

The primary basis for ARDRA is the variance in DNA sequence found in the 16S rRNA genes amplified by PCR. Tetracutter restriction endonucleases, such as Alu I and Hae III, are typically used to digest the PCR product generated from environmental DNA in ARDRA investigation. The restricted fragments are then resolved on agarose or polyacrylamide gels. The kind of microorganisms present in the environmental sample is not well-represented by ARDRA, although it can be used to compare the diversity of microbes in response to changing environmental conditions or to quickly monitor the microbial communities in environmental samples over time (Bharagava et al., 2019).

Therefore, the recent study aimed to determine the prevalence of *Salmonella sp.* in the raw and heat-treated milk from microbiological, biochemical and molecular perspective, assess its antimicrobial profile and characterize its virulence- associated genes.

Estimate the sensitivity of conventional and molecular techniques to detect *salmonella sp.* in contaminated milk samples.

Materials and Methods

1. Ethical approval

No ethical approval was required in this study. However, samples were collected as per standard sample collection methods.

2. Samples Collection

Totally 130 samples were collected from different Egyptian governorates and examined randomly. Fifty (RC-1, RC-2, RC-3, RC-4, RC-5, and So on so forth) were raw cow milk and other fifty (designated as RB-1, RB-2, RB-3, RB-4, RB-5, and So on so forth) were raw buffalo milk bought from different vendors and local daily markets. Of the remaining thirty, ten (P-1, P-2, P-3, and So on so forth) were pasteurized milks each from different brand and the other twenty (U-1, U-2, U-3, and So on so forth) were UHT- processed also from different brands.

2.1 Raw milk samples

A total of 100 retail raw cow's and buffalo's milk samples were purchased from vendors and local daily markets in different areas in Egypt and collected from October 2021 to August 2022.

The samples were collected during sampling aseptically and then put in sterile screw capped bottle (50 ml), labeled, stored by keeping in an ice box containing ice packs. Further, all samples were taken immediately to microbiology laboratory for bacteriological analysis adopting all possible hygienic measures during collection, transportation and processing of samples.

2.2 Heat treated milk samples:

Commercial dairy companies collected milks from the farmers or dairy farms, then process it via pasteurization or UHT treatment and the processed milk was packaged to sell in shops under specific brand name.

In this study, a total of 20 heat- treated milk (Pasteurized, UHT) samples were purchased from different shops. The samples were chosen randomly and collected in the usual form (plastic packets), instantly transported to the laboratory maintaining cold state and examined immediately.

3. Preparation of collected Samples:

The collected samples of *salmonella sp.* were prepared and cultured according to American Public Health Association (APHA) (Salfinger and Tortorello, 2015). Furthermore, the isolation methods are generally regarded as the standard procedure for pathogen detection followed by serological or molecular tests for confirmation (Amézquita-López et al., 2018).

4. Isolation and cultural characteristics of Salmonella sp.

Homogenized raw milk sample of 1 ml was added to 9 ml of sterilized buffered peptone water and incubated overnight at 37°C. The selective enrichment 0.1ml of pre-enrichment was transferred to 10 ml of Rappaport Vassiliadis Soya broth (RVS broth) then were incubated at 41.5 ºC for 24 hrs. Each selective enrichment broth bottle was well shaken and a loop full from each was streaked onto plates of Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric agar (HE) agar. All plates were incubated at 37°C for 24 hrs. After incubation, the XLD and HE agar plates were examined for the presence of Salmonella colonies. Moreover, colonies were transferred to nutrient agar for further tests (Gebeyehu et al., 2022; Dadi et al., 2020; Elafify et al., 2019).

5. Morphological test

According to Bano et al., 2020 microscopic test was performed for morphological characteristics of *salmonella sp*. and gram staining technique. For further confirmation several biochemical tests were performed.

6. Biochemical identification

The suspected isolates were identified biochemically using the catalase, oxidase and IMViC group ("I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test) of biochemical tests (Sedeik et al., 2019).

7. Antimicrobial susceptibility test

The antimicrobial susceptibility test of the confirmed bacterial isolates was determined using disc diffusion technique on Muller-Hinton agar (MHA) plates following the recommended Clinical and Laboratory Standard Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Kahlmeter et al., 2006). In the current study, the commercial antibiotic discs (Chloramphenicol (C30) 30µg, Amoxicillin/clavulanic acid (AMC 30) 20/10µg, Erythromycin (E15) 15µg and Cephalexin (CL30) 30µg (Bioanalyse, Turkey) were used.

The bacterial culture was spread uniformly distributed on the Muller–Hinton agar surface. The antibiotic discs were put on the surface of the inoculated plate. Then, the plates were incubated at 37◦C for 18 to 24 hours and checked for the growth of bacteria around the antibiotic discs then the data was recorded. Therefore, the antimicrobial discs, their concentrations and the diameters of the zones of inhibition for the tested strains are demonstrated according to the guidelines of EUCAST (Onohuean and Igere, 2022; Humphries et al., 2021).

8. Molecular identification and sequencing method

The molecular identification via 16S rRNA and sequencing methods were used to all suspected isolates in this study.

8.1. DNA extraction

The pure colonies of *Salmonella sp.* were used to extract genomic DNA using i- genomic BYF DNA extraction mini Kit (iNtRON, Korea), according to the manufacturer's recommendations.

8.2. DNA Quantification by NanoDrop

The purity of genomic DNA was estimated using NanoDrop (Thermo Fisher Scientific, USA). The eluted genomic DNA was stored at −20˚C for the molecular identification of the isolates.

8.3 PCR Amplification of 16S rRNA gene

To identify the *salmonella sp*. isolates, the conventional PCR amplification was performed using the full length of 16S rRNA gene of *Salmonella sp*. The specific primers of 16S rRNA gene were used. The sequence of *SA-16sS rRNA-F* is forward primer 5`-GGAACTGAGACACGGTCCAG-3` and the sequence of *SA-16S rRNA-R* is reverse primer 5`-CCAGGTAAGGTTCTTCGCGT-3`. The PCR product amplified using PCR thermocycler (SensoQuest, Germany) at 95°C for 5 min- 94°C for 1 min, 60°C for 1 min, 72 °C for 1min and 72°C for 10 min for 35 cycles. The PCR products were subjected to 1.5% agarose gel electrophoresis by running at 150V for 30 min and visualized by a gel imager using a commercial imaging system (BioRad) (Bano et al., 2020; Kaabi et al., 2019).

9. PCR Purification and sequencing

To purify 16S rRNA gene, the Montage PCR Clean UP-kit (Millipore, Germany) was used by following the manufacturer instructions. Furthermore,

the Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystems, USA) was used to purify the 16S rRNA PCR products for sequencing. As well as the 16S rRNA sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA) at the Macrogen, Inc., Seoul, Korea. The 16S rRNA sequences were analyzed using the online bioinformatics software BLAST N (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Subsequently, the 16S rRNA sequences was submitted to the National Centre for Biotechnology Information NCBI database under accession number OR048345 (https://www.ncbi.nlm.nih.gov/nuccore/OR048345) and OR102504 (https://www.ncbi.nlm.nih.gov/nuccore/OR102504.1/). Thus, the phylogenetic tree was constructed and the evolutionary relationships among salmonella and their ancestor species were evaluated by Mega11.

10. Detection of Virulence gene Profiles analysis

The virulence genes of *Salmonella sp*. ***(invA, spvA and orgA)*** and ***(sifA, iroN, and spiA)*** were determined by performing the PCR and Multiplex PCR techniques respectively using the reported primers (Table 1). The PCR products were amplified using PCR thermocycler (SensoQuest, Germany). Further, protocols for each PCR reaction were programmed following the standard protocol (Tables 2).

The PCR reactions were carried out in a total volume reaction of 25 µl that consists of 12.5 µl Master Mix (GeneDireX, Cat. No. MB203-0100), 1 µl (10 Pml) of each forward and reverse primers, and 2 µl of DNA template which extracted from suspected salmonella colonies. Deionized distilled nuclease free water was used instead of DNA template as negative control and S. Typhimurium (ATCC 14028) was used as positive controls. The PCR products were subjected to 1.5% agarose gel electrophoresis by running at 150V for 30 min and visualized by a gel imager using a commercial imaging system (BioRad) (Alzwghaibi et al.,2018).

Table 1: Primers of Virulence genes used in Multiplex PCR for identification of salmonella sp. Isolates.

Number of primer	Primer	Target gene	Length	Sequence (5′–3′)	Product size(bp)	References
1	*SA-invA-F*	invA	21	TTCCGCAACACATAGCCAAGC	95	Liang et al., 2019
	SA-invA-R	invA	23	AATCCAACAATCCATCAGCAAGG	95	
2	*SA-orgA-F*	orgA	24	TTTTTGGCAATGCATCAGGGAACA	255	Skyberg et al., 2006
	SA-orgA-R	orgA	21	GGCGAAAGCGGGGACGGTATT	255	
3	*SA-iroN-F*	iroN	26	ACTGGCACGGCTCGCTGTCGCTCTAT	1205	
	SA-iroN-R	iroN	25	CGCTTTACCGCCGTTCTGCCACTGC	1205	
4	*SA-sifA-F*	sifA	23	TTTGCCGAACGCGCCCCCACACG	449	
	SA-sifA-R	sifA	30	GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	
5	*SA-spiA-F*	spiA	30	CCAGGGGTCGTTAGTGTATTGCGTGAGATG	550	Carvalho et al., 2020; Ed-Dra et al., 2019; Skyberg et al., 2006
	SA-spiA-R	spiA	30	CGCGTAACAAAGAACCCGTAGTGATGGATT	550	
6	*SA-spv A-F*	spvA	18	GTCAGA CCC GTA AAC AGT	641	Gebreyes et al., 2009
	SA-spvA-R	spvA	18	GCA CGC AGAGTA CCC GCA	641	

Table 2: PCR conditions used for detection of Virulence genes.

	Primary denaturation	Denaturation	Annealing	Elongation	Latest elongation	No. of cycles
PCR conditions for *invA*						
Amplification protocol	94°C	94°C	60.1°C	72°C	72°C	35
Time	4 min	1min	1min	1min	10 min	
PCR conditions for *spvA*						
Amplification protocol	95°C	95°C	54°C	72°C	72°C	30
Time	5 min	1 min	1 min	1 min	10 min	
Multiplex PCR conditions for (*sifA, iroN, spiA*)						
Amplification protocol	95°C	94°C	66.5°C	72°C	72°C	25
Time	5 min	30 sec	30 sec	2 min	10 min	
PCR conditions for *orgA*						
Amplification protocol	94°C	94°C	62.3°C	72°C	72°C	35
Time	4 min	1 min	1 min	1 min	10 min	

11. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA technique is a PCR-RFLP technique referring to restriction enzyme digestion of PCR-amplified 16S rDNA gene. It exploits the sequence variation present on the 16S rDNA gene to generate a fingerprint (Dash and Das, 2018).

The in silico-ARDRA (amplified rDNA restriction analysis) was performed to identify different genus and species of the Enterobacteriaceae family isolated from the raw caw and buffalo milk (Bharagava et al., 2019; Messaoudi and Wagenlehner, 2010).

Genomic DNA was extracted and 16S rDNA gene sequence was amplified as mentioned in (Table 1-2) for 9 isolates. Furthermore, The PCR product was purified by Montage PCR Clean UP-kit (Millipore) following the manufacturer instructions. The 16S rDNA PCR products were purified and digested separately with *Alu I, EcoRI* and *Bsp12861* as restriction endonucleases enzymes. These restriction enzymes were selected on the basis of in silico analysis using the nucleotide sequence of the whole 16S rDNA gene of different *salmonella* strains, deposited in GenBank. The final reactions were carried out by following the manufacturer which recommend that PCR products were digested in the presence of 2 µl of the appropriate buffer, 10 µl of the PCR product and 2 µl of restriction enzymes (10 U / µl) then it left to react at 37 °C (for *Alu I, EcoRI* and *Bsp12861*) for 4 h and analyzed by horizontal electrophoresis in 2.5% agarose gels. The gels were visualized and gel images were digitalized (Dec et al., 2016; Messaoudi and Wagenlehner, 2010).

12. Quantitative real time PCR (qRT- PCR)

A mimic experiment was performed to make artificial contamination with salmonella sp. to sterilized milk to evaluate the capacity of qRT- PCR as a rapid and reliable diagnostic tool to detect salmonella sp. after 12, 24 and 36 hours from artificial contamination.

qRT- PCR was carried out using Maxima SYBR Green/ROX qPCR master mix (2×) (Thermo Scientific, USA) and 0.1 ml qPCR strip tubes with optical caps (Gunster Biotech Co., Taiwan). The experiment was performed using AriaMx Real-Time PCR (Agilent Technologies, USA) in triplicate technique and non-template control (NTC) (Table 3) (Liang et al., 2019; Zhou et al., 2017). Fluorescent amplification curve data were downloaded from AriaMax PCR software and analysed using LinRegPCR software.

Table 3. Oligonucleotides used for qRT-PCR analysis.

Primer	Target gene	Length	Sequence (5′–3′)	Product size (bp)	Reference
SA-invA-F	invA	21	TTCCGCAACACATAGCCAAGC	95	Liang et al., 2019; Zhou et al., 2017
SA-invA-R	invA	23	AATCCAACAATCCATCAGCAAGG		

Results and Discussion

A serious issue for public health is foodborne illness. This deeply worried the Fifty-third World Health Assembly (WHA), which in 2000 passed a resolution urging the WHO and its Member States to acknowledge food safety as a crucial public health role.

A foodborne illness can seriously harm one's health. The consumption of contaminated food causes hundreds of millions of illnesses and deaths each year. Foodborne illness affects development as well as health. Foodborne illness outbreaks have generated media coverage and consumer anxiety on a number of occasions. The main issues, meanwhile, are concealed by the enormous number of infrequent instances and little outbreaks. It is challenging to estimate the true burden of disease in a realistic manner since most nations lack adequate reporting systems. According to WHO estimates, contaminated food and/or water are the primary cause of 2, 1 million diarrhea-related deaths globally. Even in affluent nations, up to one-third of the population is thought to contract a foodborne illness each year.

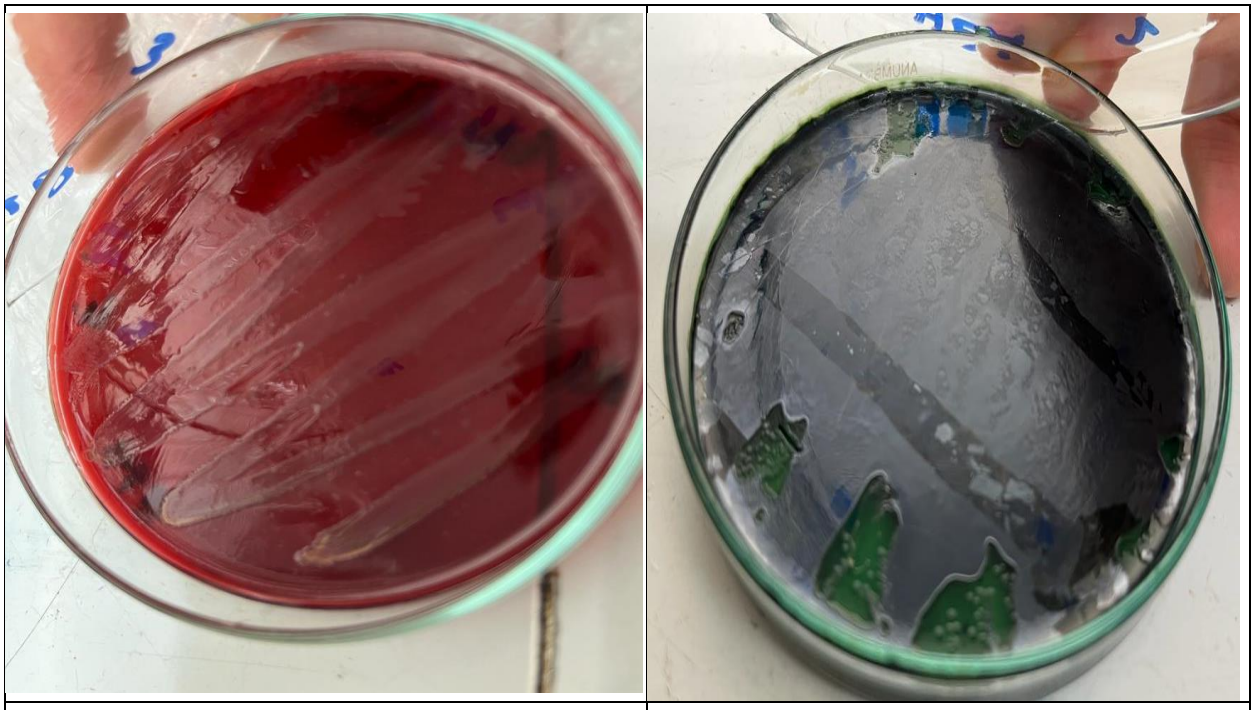
Animal source foods are considered to be the main sources of foodborne salmonellosis. Therefore, the detection of Salmonella in foods is routinely and considered as a serious part of public health programs (Gebeyehu et al., 2022).

Importantly, several illnesses can be transmitted by the raw dairy products consumption as these products are contained high prevalence of Salmonellosis and pervasiveness of *Salmonella spp*. Moreover, isolation and characterization of these food-transmitted pathogens has a great importance for the prevention and control of the disease prevalence (Dawood Saleem et al., 2022).

3.1 Cultural characteristics of *Salmonella sp.*

Table four showed that four positive samples from fifty raw caw's milk samples (8%) and five positive samples from fifty raw buffalo's milk (10%) on XLD and HE agar (Figure 1) while heat treated milk shows

negative results on XLD and HE agar which agreed with Gebeyehu et al., 2022 and Dadi et al., 2020, Who explained, typical *Salmonella sp.* colonies are pink colonies with or without black centers on XLD agar and it produces transparent green or blue-green colonies with or without black centers and appears as almost completely black colonies on HE agar.



***Salmonella sp.* on XLD agar** | ***Salmonella sp.* on HE agar**

Figure 1. Cultural characteristics of *Salmonella sp.* on XLD and HE agar.

Table 4: Incidence of *Salmonella sp.* in raw and heat-treated milk samples on XLD and HE agar.

Sample Types	samples Numbers	Number of positive samples	Incidence %
Raw cow milk	50	4	**8 %**
Raw buffalo milk	50	5	**10 %**
Heat-treated milk (pasteurized)	10	None	**None**
Heat-treated milk(UHT)	20	None	**None**
Total	130	9	**6.92 %**

3.2 Microscopic investigation for *Salmonella sp.*

The morphological characteristics of *Salmonella sp.* under microscope is pink rods (Figure 2). This indicates that it is a Gram-negative bacteria which match with the description of Parija, 2023.

3.3 Biochemical identification

The suspected isolates were identified biochemically using catalase, oxidase and IMViC group of biochemical tests. Therefore, our results showed that all *Salmonella sp.* isolates are negative to indole, Voges Proskauer and oxidase test, while they are positive to methyl red, catalase and citrate utilization test (Table 5 and Figures 3,4,5,6 and 7).

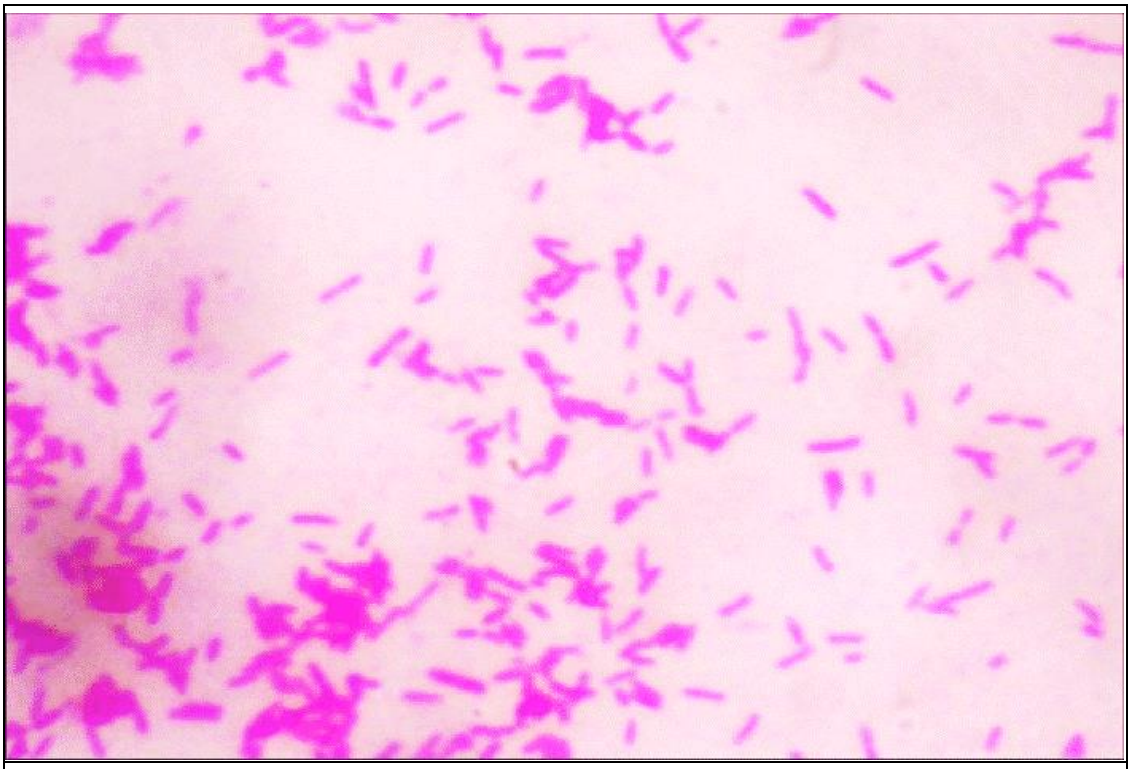


Figure 2: Microscopic investigation for *Salmonella sp.*

Table 5: Results of biochemical tests with suspected *Salmonella sp.* isolates:-

Biochemical tests	*Suspected Salmonella* isolates
Indole	−ve
Methyl red	+ve
Voges Proskauer	−ve
Citrate utilization test	+ve
Oxidase test	−ve
Catalase test	+ve

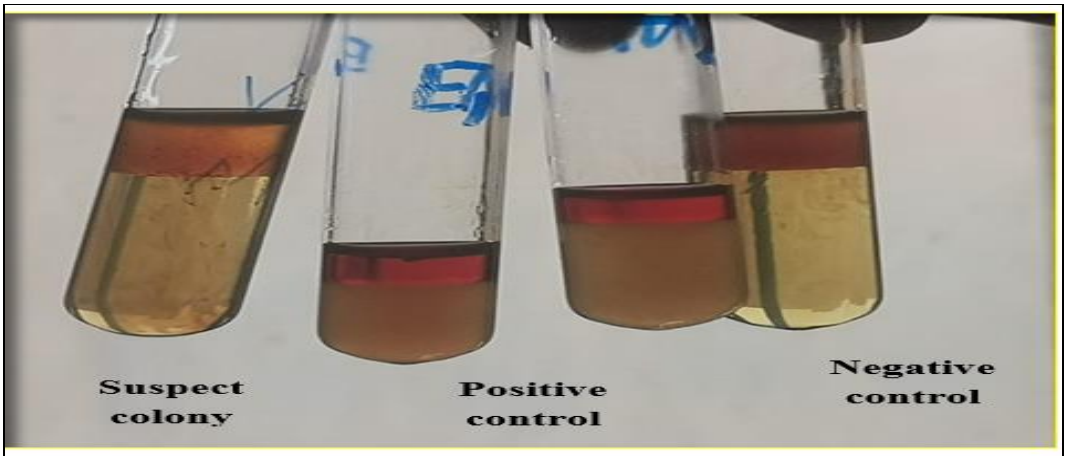


Figure 3: Results of Indole Test

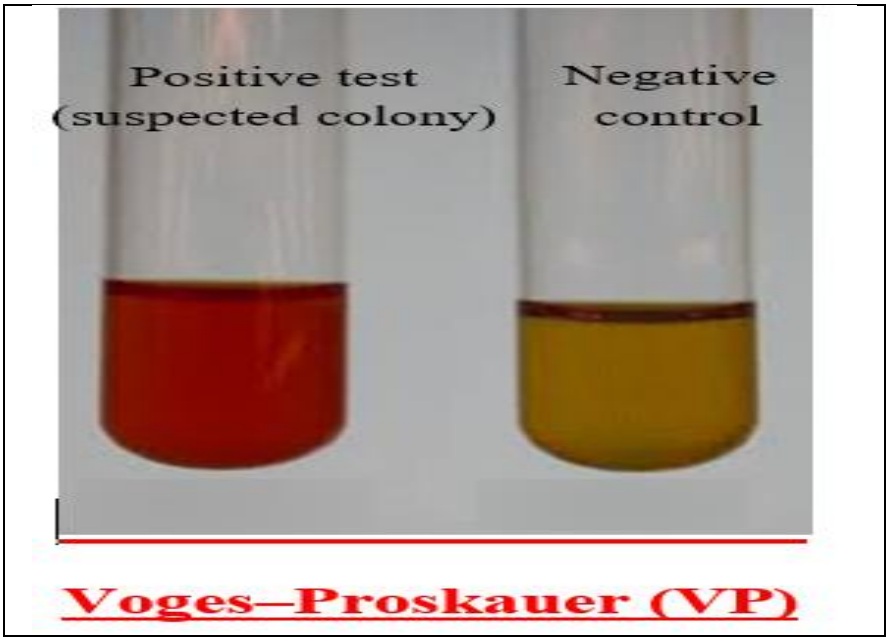


Figure 4: Results of Voges–Proskauer (VP) test.

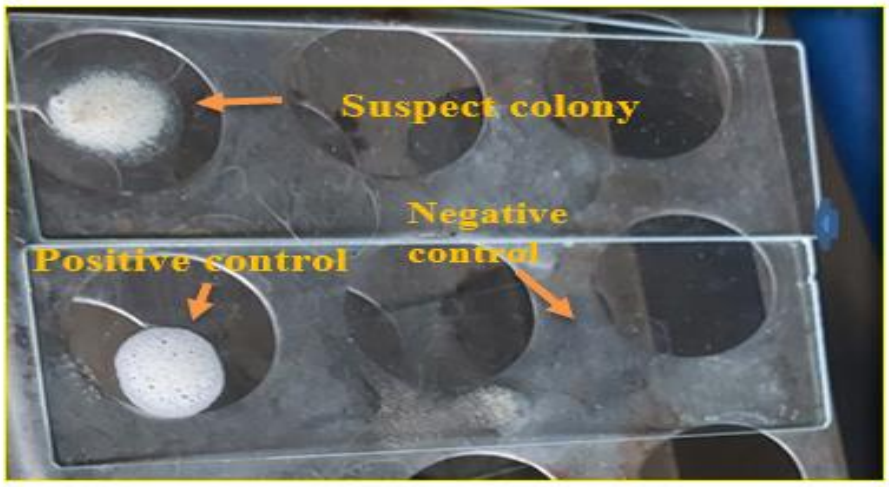


Figure 5: Results of catalase test.

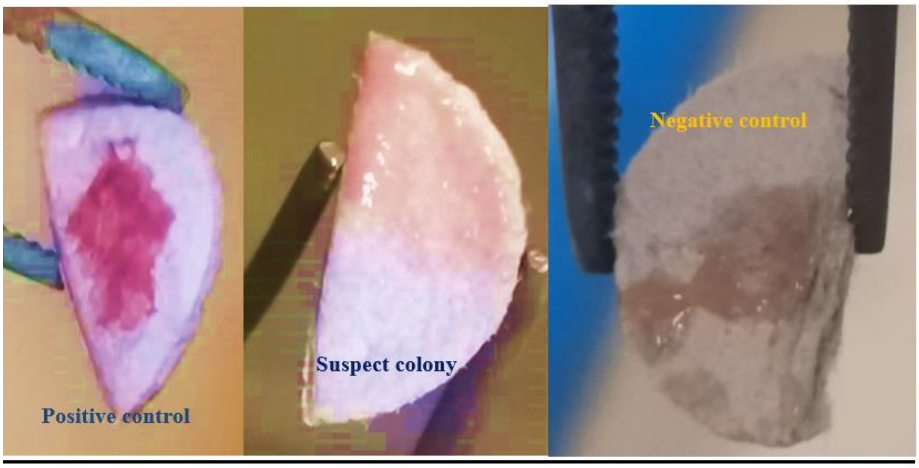


Figure 6: Results of oxidase test.

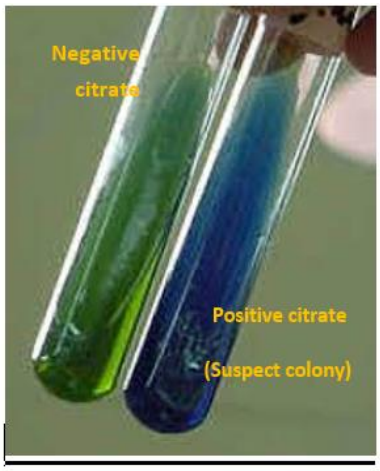


Figure 7: Results of citrate test.

3.4 Antimicrobial susceptibility test result

The antibiotic susceptibility tests of the Salmonella isolates were performed using disc diffusion technique on Muller-Hinton agar (MHA) plates according to CLSI guidelines (Elafify et al., 2019). All nine Salmonella isolates were tested for the antibiotic susceptibility profiles against four commonly used antimicrobials [Chloramphenicol (C30), Amoxicillin/clavulanic acid (AMC 30), Erythromycin (E15) and Cephalexin (CL30)]. Our results showed that the isolates were 100% sensitive to C30 (30µg). On the other hand, the all of isolates were resistant to AMC 30 (20/10µg), E15 (15µg) (Sobur et al., 2019) and CL30 (30µg) (Figure 8, Table 6).

Therefore, the three antimicrobial resistances were checked and recorded 100% sensitivity in all isolates (Table 7).

Figure 8: Result of antimicrobial susceptibility test.

Table 6. Antimicrobial susceptibility test in *Salmonella sp*.

Antibiotic tested	Status of antimicrobial agent against the isolates		
	Resistant (%)	**Intermediate (%)**	**Susceptible (%)**
Chloramphenicol(C30)	—	—	9 (100%)
Amoxicillin/clavulanic acid (AMC 30) 20/10µg	9 (100%)	—	—
Erythromycin (E15) 15µg	9 (100%)	—	—
Cephalexin (CL30) 30µg	9 (100%)	—	—

Table 7. The three antimicrobial susceptibility test in *Salmonella sp.* isolates

Antimicrobial Resistance Number	Patterns of antimicrobial Resistance (isolates number)	Isolates Number (%)
Three	1. Amoxicillin/clavulanic acid (AMC 30) 20/10µg 2. Erythromycin (E15) 15µg 3. Cephalexin (CL30) 30µg	9 (100%)

3.5 Molecular characterization of *salmonella sp.*

Polymerase chain reaction (PCR) is the most widely molecular tools that used for the rapid and reliable detection and molecular identification of bacterial isolates.

16Sr RNA PCR is a rapid standard PCR technique for bacterial identification on genus levels.

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy because it includes:-

1. Its presence in almost all bacteria, often existing as a multigene family, or operons.
2. The function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution).
3. The 16S rRNA gene (1,500 bp) is large enough for informatics purposes.
4. The full length of 16S rRNA gene is 660 bp compared to DNA marker (100 bp DNA ladder) as shown in (Figure 9). Our results confirmed that all isolates including 16S rRNA with molecular weight 660 base pair as previous published data (Bano et al., 2020; Kaabi and AL-Yassari 2019; Rosselli et al., 2016).

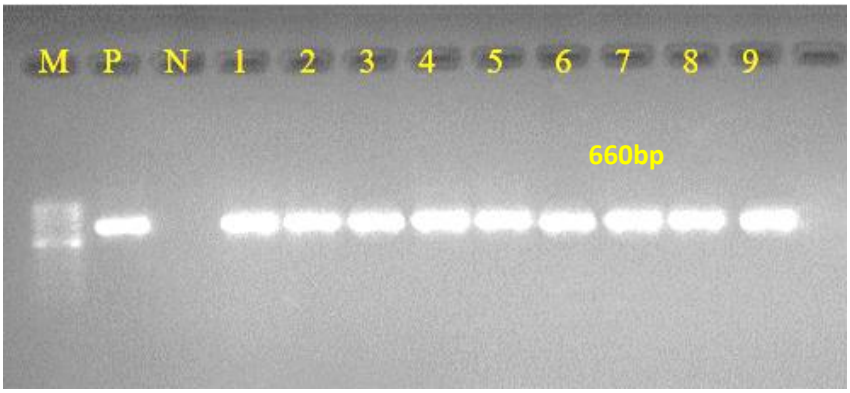


Figure 9: Electrophoresis result of PCR products of 16S rRNA gene of *Salmonella sp.* M indicated to the molecular marker of 100 bp DNA ladder; Lane P indicated to the positive control (*S. typhimurium* (ATCC 14028); Lane N indicated to the negative control; Lane 1-9 indicated to the PCR products of 16S rRNA genes of *Salmonella sp.* isolated from raw milk.

3.6 Result of ARDRA technique

To differentiate among species of nine isolates, which appear that they belong to *Salmonella sp.* according to morphological, biochemical tests and cultural characteristics, we use amplified ribosomal DNA restriction analysis (ARDRA) technique by using a panel of three enzymes already proven suitable for the identification of salmonella species. Our results of ARDRA technique revealed that all isolates belonging to *Salmonella enterica.* Based on the ARDRA profile, Alu I and Eco RI were produced two fragments with molecular weights (532bp, 90bp, 346bp and 276bp, respectively) (Figure 10. a ,b), while Bsp12861 are yielded four ARDRA patterns (Figure 10. c).

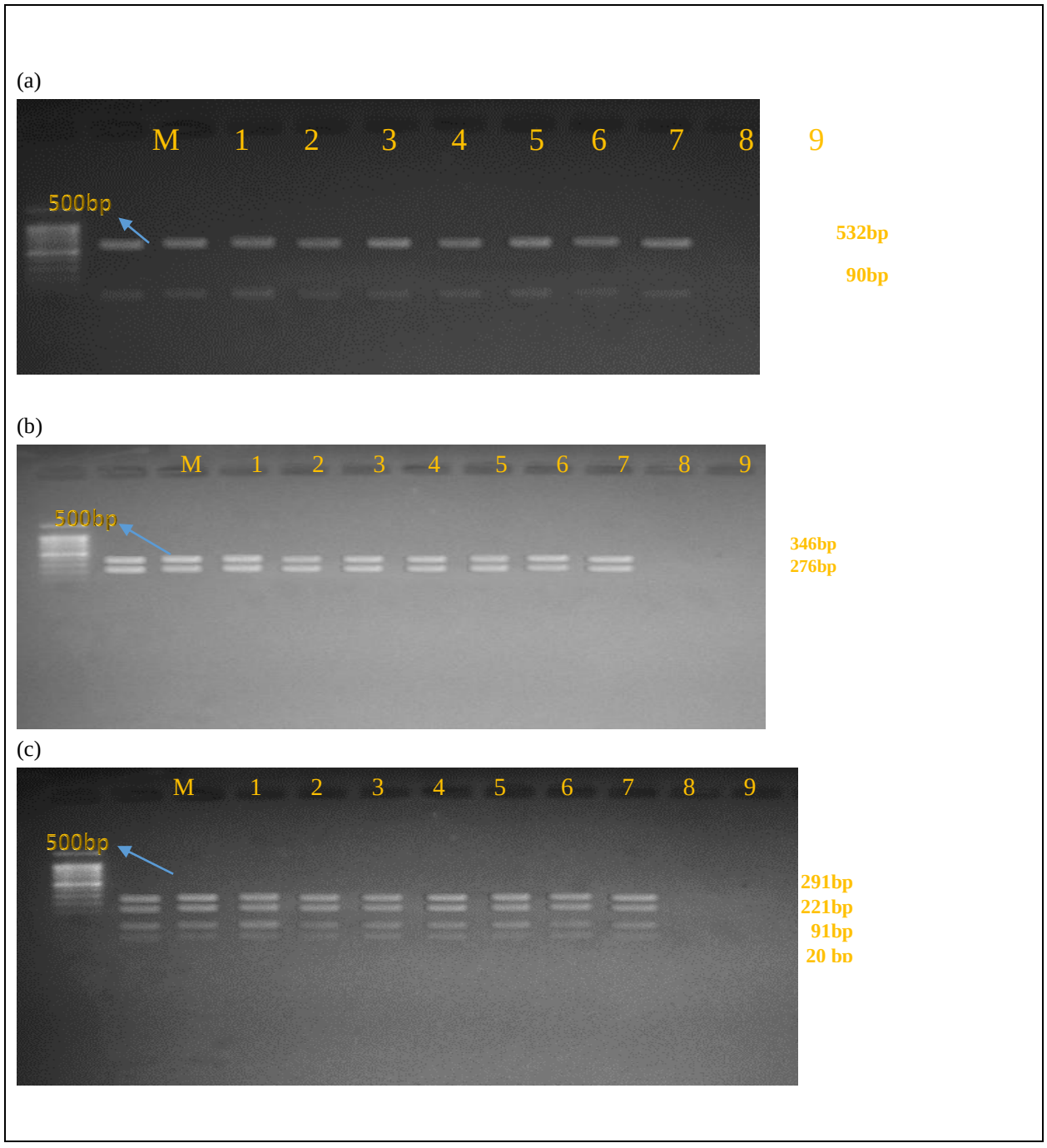


Figure 10: Amplified Ribosomal DNA Restriction Analysis (ARDRA) technique was carried out using three restriction enzymes of 16S rRNA gene for 9 isolates; (a) ARDRA using Alu I restriction enzyme; (b) ARDRA using EcoRI restriction enzyme, (c) ARDRA using Bsp12861 restriction enzyme; M indicated to the molecular marker of 100 bp DNA ladder, numbers from 1 to 4 indicated to isolates from raw cow milk while numbers from 5 to 9 indicated to isolates from raw buffalo milk.

3.7 Nucleotides sequencing sets

The PCR, DNA cloning and sequencing, especially the 16S rRNA gene sequencing plays important role in the molecular identification of bacterial isolates and the novel species discovery (Kaabi and AL-Yassari, 2019). Moreover, the 16S rRNA gene is a significant marker in the evolution and classification studies of living organisms. Therefore, the sequencing of 16S rRNA gene is a rapid method for unusual phenotypic bacterial identification. The nucleotides sets findings were analysed and confirmed by using BLAST analysis.

Importantly, the 16S rRNA gene sequence databases at GenBank has been widely used for the molecular identification of unknown bacteria up to the genus or species level (Fadlalla et al., 2021; Kaabi and AL-Yassari, 2019; Srinivasan et al., 2015). Therefore, the isolates of *Salmonella sp.* were registered in NCBI based on sequencing of 16S rRNA gene with the accession numbers: (OR048345, OR102504).

The BLAST sequencing results, multiple sequence aliment (MSA) (Figures 11, 12) and phylogenetic trees were conferred that 16S rRNA nucleotide sequence of OR048345 is similar to *Salmonella enterica* subsp. enterica serovar Enteritidis strain HK10 16S ribosomal RNA gene, partial sequence, *Salmonella enterica* strain HHL34W 16S ribosomal RNA gene, partial sequence, *Salmonella enterica* subsp. enterica serovar Kentucky strain HHL31W 16S ribosomal RNA gene, partial sequence and *Salmonella enterica* subsp. enterica serovar Typhimurium strain H1 16S ribosomal RNA gene, partial sequence under accession numbers MH109386.1,OP683357.1, OP683354.1, MH109313.1 respectively with 100% identity while OR102504 is similar to *Salmonella enterica* subsp. enterica serovar Enteritidis strain HK10 16S ribosomal RNA gene, partial sequence, *Salmonella enterica* strain NMSF15 16S ribosomal RNA gene, partial sequence, *Salmonella enterica* strain HHL34W 16S ribosomal RNA gene, partial sequence and Salmonella enterica subsp. enterica serovar Kentucky strain HHL31W 16S ribosomal RNA gene, partial sequence under accession numbers MH109386.1, OR048345.1, OP683357.1, OP683354.1,OK161095.1 respectively with 97.86% identity.

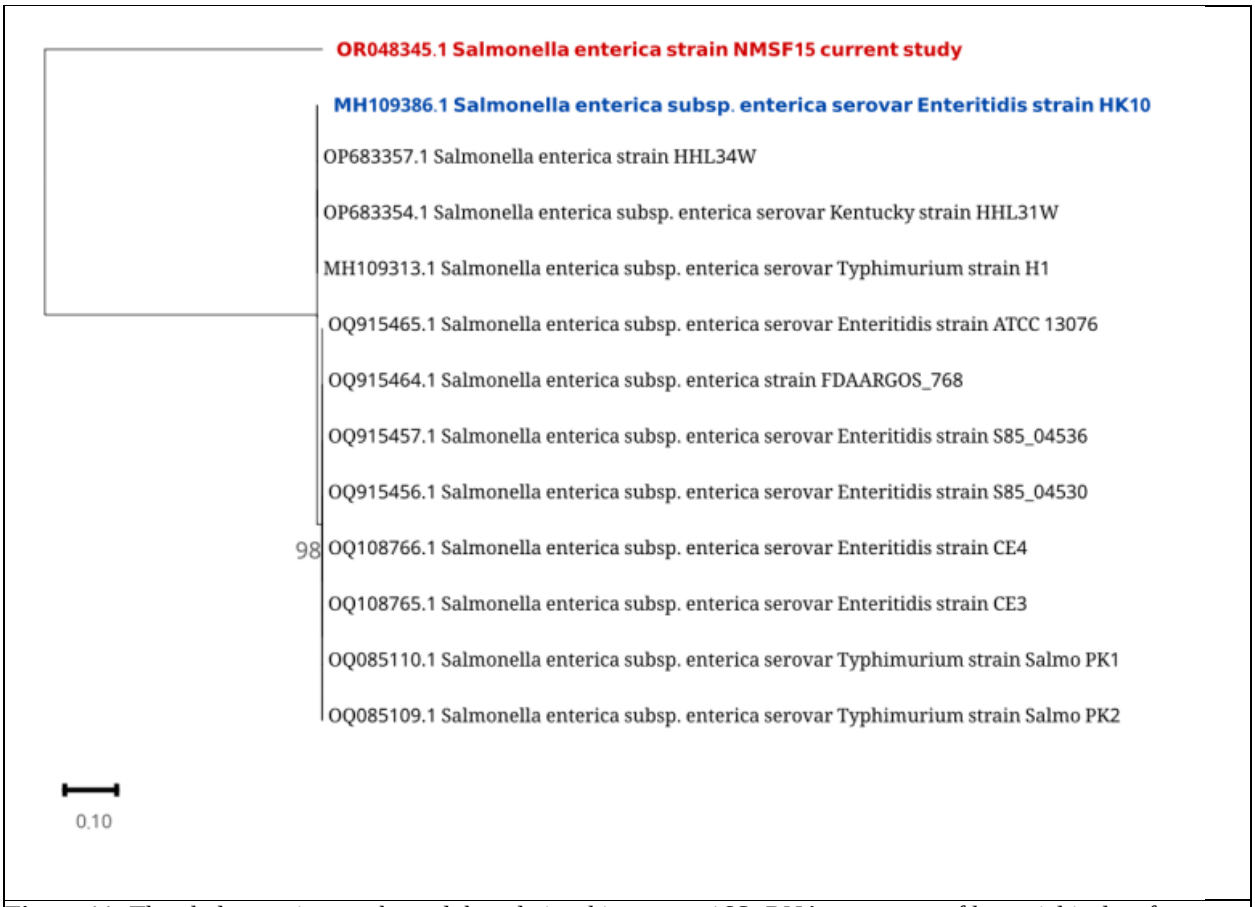


Figure 11: The phylogenetic tree showed the relationship among 16S rRNA sequences of bacterial isolate from caw raw milk.

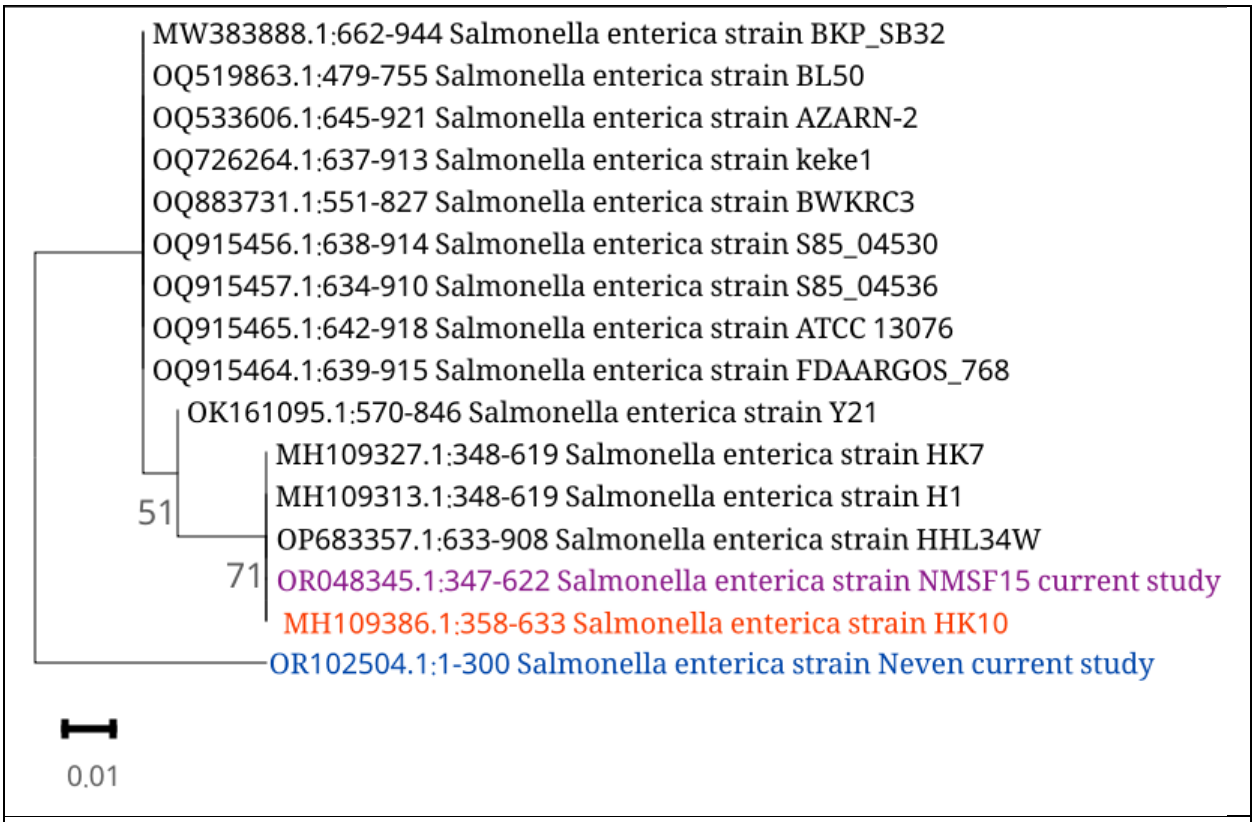
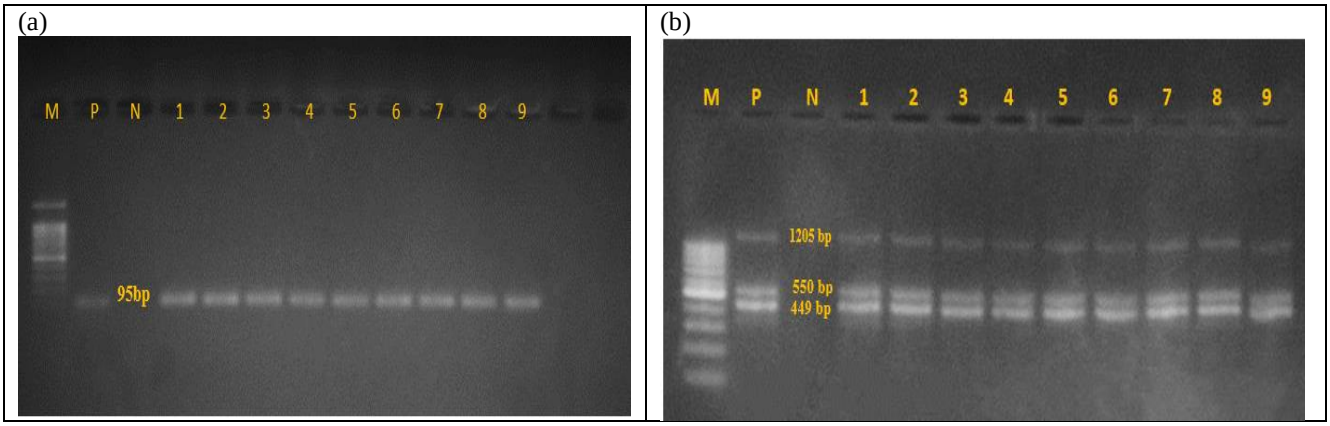


Figure 12: Neighbor- joining tree showed the phylogenetic relationship of OR102504 belonging to *Salmonella enterica*.

3.8 Genomic Virulence Genes Detection in Salmonella sp.

Our results correspond to the results of Liang et al., 2022; Liang et al., 2019; Zhou et al., 2017, Fu et al., 2022 and Buehler et al., 2019. Further, our result showed that all isolates have *iro N*, *spi A*, *sif A* genes with molecular weight 1205, 550, 449 respectively (figure 13 b) and these results agreed with Skyberg et al., 2006. Additionally, our results showed that all isolates have *spvA* and *orgA* virulence genes in all salmonella isolates (figure 13 c and d). The virulence genes were revealed by using PCR (Figure 13). Therefore, the isolates were confirmed as *Salmonella* by detecting the specific gene *invA* (Figure 13 a) by simplex PCR because *invA* has been reported to have mutation rates similar to housekeeping genes, suggesting it is a suitable candidate for PCR-based detection assays (Buehler et al., 2019).



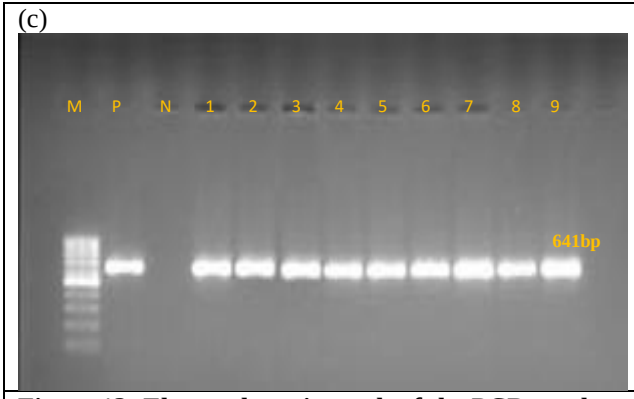


Figure 13: Electrophoresis result of the PCR products of virulence genes of Salmonella sp. (a) indicated to the PCR products of *invA* gene of *Salmonella sp.* ;(b) indicated to the Multiplex PCR products of *iro N*, *spi A*, *sif A* virulance genes of *Salmonella sp*.; (c) indicated to the PCR products of *spv A* gene of *Salmonella sp*.; (d) indicated to the PCR products of *org A* gene of *Salmonella sp.* ; M indicated to the molecular marker of 100-bp DNA Ladder; Lane P indicated to the positive control (*S. typhimurium* (ATCC 14028); Lane N indicated to the negative control; numbers from 1 to 4 indicated to amplifications of virulance genes of *Salmonella sp.* isolated from raw caw's milk while numbers from 5 to 9 indicated to the amplifications of virulance genes of *Salmonella sp.* isolated from raw buffalo's milk.

3.9 qRT- PCR in detecting *Salmonella sp.*

Real-time PCR, commonly referred to as quantitative PCR (qPCR), is a technique that quantifies the amount of template DNA in a sample and detects the PCR result in real-time without agarose gel electrophoresis. It uses similar DNA amplification conditions as conventional PCR. Because qPCR is applicable to both quantitative and qualitative analysis, it is more dynamic and versatile for a wider range of applications. These advancements significantly shorten the time needed to determine and count the number of microorganisms present in food. Foodborne pathogens can be quantified quickly and precisely with qPCR. The fluorescent reporter/dye used in qPCR has the ability to emit fluorescence in direct proportion to the amount of amplified DNA product. Fluorescent molecules can be employed in qPCR as fluorescently labeled probes like TaqMan or as DNA binding dyes like SYBR Green. In terms of identifying and measuring target DNA, the former is more precise than the latter. To estimate the amplicon, the fluorescence produced in each qPCR cycle will be measured and quantified (Bio-Rad, 2020).qPCR has been reported to give comparable results to those obtained using plate counts. As such, it has been suggested as an alternative to TVC that can provide accurate results in a shorter time. Recent study has shown multiplex qPCR to be capable of simultaneously detecting up to 12 important foodborne pathogens in a single assay (Tang, 2023).

According to Heymans et al., 2018 qRT-PCR was performed using specific primer (*invA*) gene. Therefore, the results explained that *salmonella sp.* can't be detected after 12h while can be detected after 24 (Figures 14, 15, 16).

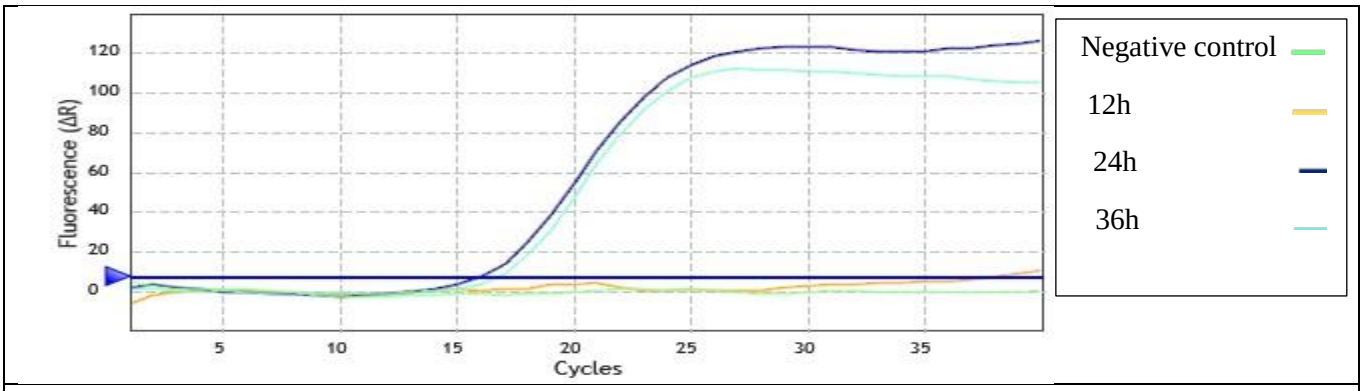


Figure 14: Amplification plots of invA gene.

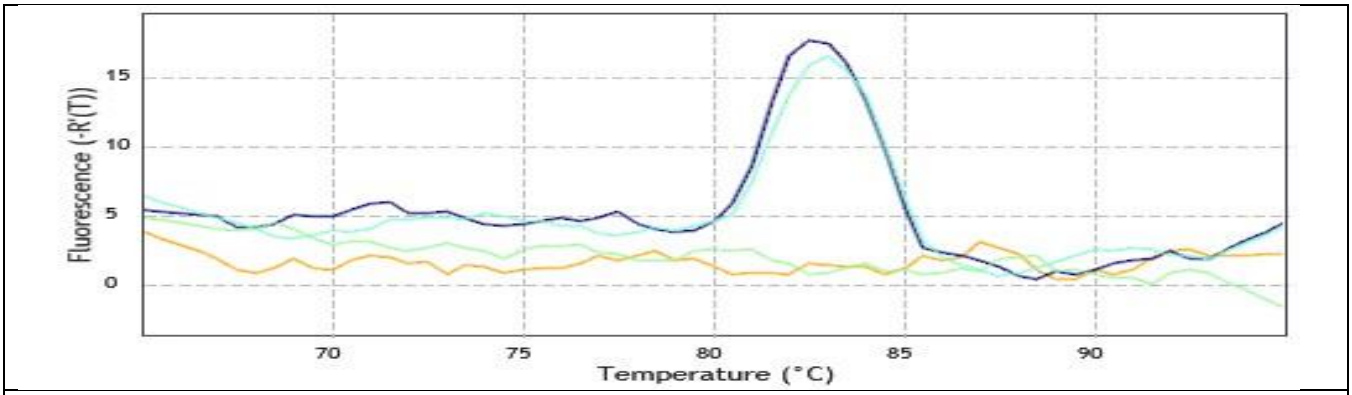


Figure 15: Melting curve of invA gene.

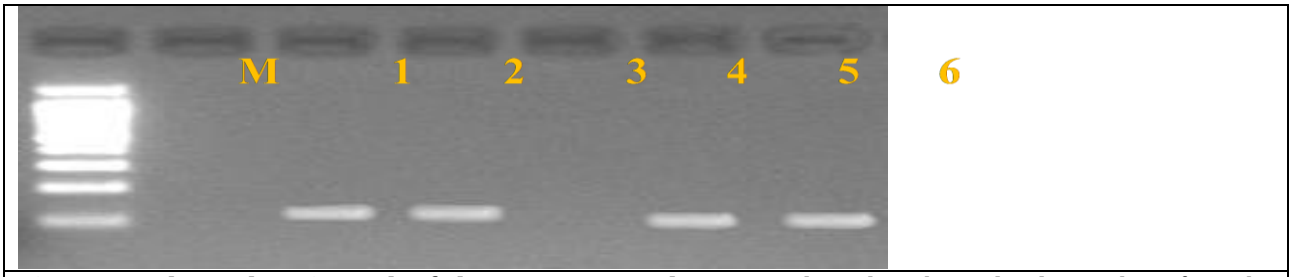


Figure 16: Electrophoresis result of the qRT-PCR products; M indicated to the molecular marker of 100-bp DNA Ladder; numbers 1, 2, F3 indicated to 16S rRNA after 12h, 24h, 36h respectively , numbers 4,5,6 indicated to invA gene after 12h, 24h,36h respectively.

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التعريف الجزيئى وخصائص السالمونيلا فى الالبان الخام والمعاملة حراريا المتواجدة بالسوق المحلى

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الحليب هو طعام مغذي و قيم وله مدة صلاحية قصيرة ويتطلب معالجة دقيقة. ولذلك فإن الحليب سريع الفساد لأنه وسط مناسب لنمو الكائنات الحية الدقيقة بشكل خاص؛ المسببات المرضية البكتيرية التي يمكن أن تسبب تلف الحليب واصابة المستهلكين. لذا هدفت هذه الدراسة إلى تحديد مدى انتشار السالمونيلا .sp في الحليب الخام والمعالج بالحرارة، وتوصيف الجينات المرتبطة بسمية البكتريا، وتقييم خصائصها المضادة للميكروبات.وفى هذه الدراسة تم استخدام مائة وثلاثين عينة (50 حليب بقري خام، 50 حليب جاموس خام، 10 حليب معالج حراريا (مبستر)، 20 حليب معالج بالحرارة ((UHT)لتقييم مدى انتشار السالمونيلا .sp وتم استخراج تسعة عزلات من السالمونيلا اربعة من حليب البقر الخام (8%) وخمسة من حليب الجاموس الخام (10%) بينما لم يتم الحصول على عزلات من السالمونيلا .sp من الحليب المعالج حراريا. علاوة على ذلك، أشار اختبار الحساسية للمضادات الميكروبية إلى أن جميع العزلات كانت مقاومة للأموكسيسيلين/حمض الكلافولانيك 20/10 (AMC 30) ميكروجرام، إريثرومايسين (E15) 15ميكروجرام وسيفالكسين 30 (CL30) ميكروجرام في حين أن جميعها حساسة للكلورامفينيكول 30 (C30) ميكروجرام. بالاضافة الى ان نتائج تفاعل البلمرة المتسلسل أظهرت أن جينات السمية الستة (*invA* ، *orgA*، *sifA*، *spvA*، *iroN* و*spiA*) تم اكتشافها بشكل معنوي في العزلات التسعة جميعها. علاوة على ذلك، أظهرت نتائج تقنية ARDRA أن جميع العزلات تنتمي إلى السالمونيلا *Salmonella enterica* وتم التحقق من صحتها باستخدام تقنية PCR تفاعل البلمرة الكمى . وبناء عليه تشير نتائجنا إلى عدم كفاية التدابير الصحية المعتمدة أثناء عملية الحلب . ولذلك، يوصى باتباع الأساليب الصحية الصارمة أثناء الحلب والمعالجة.