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Research Article

Molecular Identification, Typing, and Virulence Gene Profiling of *Salmonella* Isolates Recovered from Cattle and Buffaloes in Egypt

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Abstract

Salmonella is a major cause of diarrhea in cattle and buffaloes. This study aimed to isolate and biochemically identify *Salmonella* species from faeces of cattle and buffaloes, confirm and type isolated *Salmonella* species molecularly, determine *Salmonella* infection-associated risk factors, and detect selected virulence genes. A total of 123 fecal samples were collected from cattle and buffaloes from different Egyptian governorates. All samples were processed using standard culture methods, followed by biochemical identification using conventional methods and the VITEK-2 System, with confirmation by qPCR. Conventional PCR was also used for *Salmonella* serovar identification and detection of the virulence genes *spvB, spvC, spvR,* and *pefA*. Bacteriological examinations revealed 13 *Salmonella* isolates (10.57%); however, the VITEK-2 identification system detected only 12. The qPCR confirmed that 13 were positive for *Salmonella*. Conventional PCR demonstrated that all 13 *Salmonella* isolates were of the Typhimurium serovar. Salmonella isolation was significantly higher in diarrheic animals and in animals younger than 3 months (P<0.05), while species, sex, season, and geographic location had no significant effect (P>0.05). Out of 13 S. Typhimurium isolates, 8 isolates tested positive for *spvC*, 7 for both *spvB* and *pefA*, and 5 for *spvR*, suggesting their limited role in the pathogenesis of diarrhea. This study concluded that qPCR showed higher detection performance for *Salmonella* isolates than the VITEK-2 system.

Keywords: Cattle and buffaloes, Molecular typing, qPCR, *Salmonella*, Virulence genes, VITEK-2

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Introduction

Salmonella are asporogenic, non-capsulated, rod-shaped bacteria measuring approximately 2.0-5.0 µm in length and 0.7-1.5 µm in width. They are Gram-negative bacteria belonging to the family *Enterobacteriaceae* [1]. *Salmonella* is widely distributed among animals, humans, and the environment, enabling transmission and cross-contamination [2]. It exhibits enormous species variety, with more than 2.600 identified serovars [3]. *Salmonella enterica* is a major pathogen that impacts cattle and buffalo-rearing systems globally [4,5], with *Salmonella enterica* serovar Typhimurium being the most recognized *Salmonella* serovar in African food animals and meat products [6].

Salmonella is an infectious, contagious bacterium that causes a range of clinical problems in cattle and buffaloes, including enteritis, septicemia, pneumonia, and reproductive disorders, all of which are potential manifestations of *Salmonella* infection in cattle and buffaloes [4,7,8]. Animals, frequently being asymptomatic carriers of infections, excrete them with feces, thus distributing them to the environment [9].

Enteric disease can affect cattle of all ages, from neonates to adults [10]. Among the causative agents, *Salmonella* is a major pathogen responsible for diarrhea in both cattle and buffaloes, particularly in young calves [4,7]. Notably, the prevalence of *Salmonella* shedding decreases with

increasing age in calves [11]. *Salmonella enterica* serovar Typhimurium is associated with severe diarrheal disease, often characterized by fibrinous enteritis as a prominent postmortem finding, and contributes to substantial economic losses worldwide [4,7].

The isolation of the organism from tissues obtained aseptically during necropsy or from faeces, rectal swabs, or environmental samples, food products, and feedstuffs is the basis for diagnosis [12].

PCR-based technology can be employed for the rapid and precise detection of *Salmonella* [13]. The invasion *(invA)* gene is a virulence gene related to host recognition/invasion [5] and is also a common molecular target for *Salmonella*-specific detection methods, and the U.S. Food and Drug Administration Bacteriological Analytical Manual recommends it for PCR confirmation of *Salmonella* isolates [3].

Real-time quantitative polymerase chain reaction (qPCR), a molecular approach, has become a highly effective diagnostic alternative for the rapid and specific identification of several pathogens, including *Salmonella*. qPCR is a simple, fast (detection in less than 24 h), sensitive, specific, and repeatable technique [14].

Salmonella colonizes its hosts by invading, adhering to, and evading the host's intestinal defensive mechanisms, such as gastric acid, using different virulence markers and determinants, including flagella, capsule, plasmids, adhesion systems, and secretion systems [12]. *Salmonella's* pathogenicity is complicated, due to the presence of multiple virulence genes housed in *Salmonella* pathogenicity islands (SPIs), plasmids, and other gene cassettes. The high frequency of virulence genes in *Salmonella* indicates that the bacteria have a high potential for pathogenicity [15]. Mobile elements, such as plasmids, play an important role in the rapid evolution of *Salmonella* in adaptability to environmental challenges, allowing the survival and extensive distribution of diverse *Salmonella* serovars [16].

The *Salmonella* plasmid virulence *(spv)* region is the *Salmonella* virulence plasmids' signature locus. It contains the *spvR* gene, which encodes a LysR-like transcriptional regulator that positively regulates both the independent transcription of its own gene as well as the *spvABCD* operon. The *spvB, spvC,* and *spvD* proteins enter host cells via a type-three secretion system (T3SS) encoded by the *Salmonella* pathogenicity island-2 [17]. It has been established that *SpvB* inhibits actin polymerization by ADP-ribosylation of actin monomers, a well-recognized process during host intracellular infection [17], while *spvC* possesses phosphothreonine lyase activity and has been identified as a suppressor of MAP kinase signaling [18]. In *Salmonella* Typhimurium, the production of surface filamentous structures is determined by plasmid-encoded fimbriae locus *(pef)* genes carried on a multicopy plasmid. *Pef* promotes adherence to the small intestine [19].

In Egypt, cattle and buffaloes represent the primary sources of red meat and milk for human consumption [20,21]. Studies investigating *Salmonella* infection in Egyptian livestock, particularly buffaloes, remain limited, especially regarding its types, associated risk factors, and virulence determinants.

This study aimed to isolate and biochemically identify *Salmonella* species using conventional methods and the advanced VITEK-2 technique from the faeces of cattle and buffaloes from different locations in Egypt. Confirmation of isolated *Salmonella* species using the real-time PCR technique, and subsequent typing of confirmed *Salmonella* species using the conventional PCR technique. Assessment of *Salmonella* infection-associated risk factors. Detection of different virulence factors *(spvR, spvB, spvC,* and *pefA)* using the conventional PCR technique.

Material and Methods

Ethical Approval

The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Cairo University (Vet. CU. IACUC), with code number (Vet CU301220251286) approved all the methods of animal handling and sampling included in this study.

Animal Sampling and Data Collection

A total of 123 individual fecal samples were collected from cattle and buffalo herds across five Egyptian governorates. The sampled animals included 66 animals showing diarrhea and 57 apparently healthy animals that had been in contact with diarrheic cases. Fecal samples were obtained directly from the rectum using sterile gloves and aseptically transferred into labeled, sterile, screw-capped containers. Following collection, samples were transported to the laboratory in an icebox and stored at 4°C until processing within 24 h [22].

The collected data from the sampled animals were classified according to their health status (diarrheic and apparently healthy), location (Faiyum, Alexandria, Behera, Menoufia, and Giza), age (<3 months, 3-6 months, and >6 months), sex (female and male), and season (Summer-Spring and Autumn-Winter).

Isolation and Identification of *Salmonella*

Isolation was carried out in accordance with the previously described protocol [23]. The specimens were pre-enriched in buffered peptone water (BPW, Oxoid, UK) and incubated at 37±1°C for 18±2 h. A 0.1 mL aliquot of the pre-enrichment broth was transferred to 10 mL of Rappaport-Vassiliadis (RV, HiMedia, India) broth

and incubated at 41±1°C for 24±3 h. After incubation, a loopful of culture was streaked over the surface of xylose-lysine-deoxycholate (XLD, HiMedia, India) and hektoen enteric agar (HE, HiMedia, India) media, which were then incubated at 37°C for 24 h. The XLD and HE plates were examined for the presence of *Salmonella* colonies. The production of red colonies with black centers and black colonies was inspected on XLD and HE plates, respectively.

Presumptive colonies were selected based on their cultural and morphological characteristics in Gram-stained films and then processed for biochemical analysis, which included oxidase, catalase, urease, citrate utilization, indole, triple sugar iron (TSI), and lysine iron agar tests. A single pure colony from each isolate was subcultured on nutrient semisolid agar at 37±1°C for 24 h and preserved for further molecular analysis.

Biochemical Identification Using the VITEK-2 System

All suspected colonies were biochemically tested using the VITEK-2 system (bioMérieux, France) and the VITEK-2 Gram-negative identification cards, following the manufacturer's instructions. Briefly, three to five fresh colonies from each isolate were transferred into tubes containing 3 mL of the suspension buffer provided with the VITEK-2 cards. The OD was adjusted to ~0.8 and used to load the identification cards into the vacuum chambers. The cards were then placed in the automated laser detection chambers and left to complete the identification process and create the log file [24].

Molecular Identification and Typing of Isolated *Salmonella*

DNA Extraction: A single colony grown on XLD agar plates was picked up, inoculated into brain heart infusion (BHI, HiMedia, India) broth, and incubated overnight at 37°C with shaking. The culture was then centrifuged, and DNA was extracted from cell pellets using the EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech Co., LTD, China), following the manufacturer's instructions.

DNA Quantitation: The DNA concentration was determined using the Qubit 2.0 Fluorometer and the dsDNA BR test kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions. Briefly, 1 µL of DNA was mixed with 199 µL of the DNA dilution buffer and incubated for 3 min at room temp. The mixture's absorbance was measured, and the results were plotted against a standard curve created using the kit's standard DNA concentration.

Real-Time PCR for Detection of *Salmonella* spp.

InvA primers (F:5'-ACAGTGCTCGTTTACGACCTGAAT-3', R:5'-AGACGGCTGGTACTGATTATAAT-3') and TaqMan probe (5'-Fam-CGA-CCC-CAT-AAACACCAATATCGCC-BHQ1b-3') sequences were purchased from Metabion company (Germany). The primers were analyzed using fast PCR v6 [25] to amplify 243 bp of *Salmonella* spp. *invA* gene. They were blasted against the nucleotide database of the NCBI website to ensure identity among published BLAST sequences for the target gene and the absence of significant similarity with other microorganism sequences. The probe was labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5' end and with Black Hole Quencher R (BHQ) at the 3' end of the probe.

The qPCR was run in a Taqman mastermix containing 10 µL of EasyScript qPCR Master Mix (Agilent Technologies, USA), 250 nM of each *invA* primer, 100 nM of *invA* probe, and 2 µL of purified DNA in a final volume of 20 µL using nuclease-free water. The qPCR experiments were performed using the AriaMx real-time PCR thermocycler equipment. The optimal qPCR efficacy was achieved using a cycling profile including a denaturation at 95°C for 30 s, followed by 40 cycles of 95°C/20sec, 60°C/20sec and 72°C/30 sec. Each qPCR had one positive control (*S. enterica* DNA) and a no-template control (master mix and sterile water) [26].

Molecular Typing of *Salmonella* Serovars Using Conventional PCR

Each serovar-specific PCR assay included a positive control consisting of DNA from the corresponding target serovar and a no-template negative control. The primer sets used were selected from previously validated studies demonstrating specificity for their respective target serovars. Uniplex PCR was used for the detection of the *MDH* (malic acid dehydrogenase) gene of serovar Typhimurium [27]. Multiplex PCR was applied using three pairs of oligonucleotide primers *(tcpS*, *lygD*, and *flhBinner*) for the detection of *S.* Enteritidis and *S.* Dublin [28] *(Table 1)*.

Molecular Detection of Virulence Factors' Genes

Plasmid Extraction Using a Miniprep Purification Kit: A single colony grown on XLD agar plates was picked up, inoculated into BHI broth, and incubated overnight at 37°C with shaking, then cooled down and centrifuged at 4000 rpm/10 min at 4°C; plasmids were recovered using the miniprep purification kit (EasyPure® Plasmid MiniPrep Kit, TransGen Biotech Co., LTD, China) following the manufacturer's instructions.

Amplification of the Virulence Factors' Genes: The purified plasmids from various colonies were tested for the presence of several genes known to be responsible for virulence *(pefA, spvB, spvC,* and *spvR)* using different sets of primers using conventional PCR. The primer sets and thermal cycling conditions of different virulence genes are presented in *Table 2*.

The reaction mixture was prepared in 25 µL. It consisted

Table 1. *Primers used for the identification of S. Typhimurium, S. Enteritidis, and S. Dublin*				
Target Gene	**Primer Sequence (5' - 3')**	**Annealing Temperature (°C)**	**Amplicon Size (bp)**	**References**
MDH	F: TGCCAACGGAAGTTGAAGTG R: CGCATTCCACCACGCCCTTC	55	261	[27]
tcpS	F: ATGTCTATAAGCACCACAATG R: TCATTTCAATAATGATTCAAGC	55	882	[28]
lygD	F: CATTCTGACCTTTAAGCCGGTCAATGAG R: CCAAAAAGCGAGACCTCAAACTTACTCAG	55	339	[28]
flhBinner	F: GCGGACGTCATTGTCACTAACCCGACG R: TCTAAAGTGGGAACCCGATGTTCAGCG	55	155	[28]
MDH gene for detection of S. Typhimurium, tcpS, lygD, and flhBinner for detection of S. Enteritidis, and tcpS and flhBinner for detection of S. Dublin				

of: 12.5 µL of 2X PCR Master Mix (TransGen Biotech Co., LTD, China), 1 µL of each forward and reverse primer (20 pmol) (Metabion, Germany), 4.5 µL of PCR-grade water, and 6 µL of purified plasmids. DNAse-free RNAse-free water was used as a negative control.

Visualization of the PCR Products

Ten microliters of each PCR product were electrophoresed in 1.5% agarose gel containing 0.5 µg/mL ethidium bromide (Sigma, Germany) in 1× TBE buffer against a 100 bp DNA ladder (Qiagen, USA). The gel was then visualized and photographed with a gel documentation system (Alpha Inno tech, Biometra, USA).

Statistical Analysis

Fisher's exact test and the chi-square test were used to assess associations between the studied variables and *Salmonella* isolation rates. Statistical significance was defined as a P-value <0.05. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for each variable, with baseline categories specified in *Table 3*. All statistical analyses were performed using SPSS software, version 24 (IBM Corp., Armonk, NY, USA).

Results

Cultural, Morphological, and Conventional Biochemical Identification of *Salmonella*

Out of 123 cultured fecal samples, 13 showed colonies with morphological characteristics consistent with *Salmonella* on XLD and HE media *(Fig. 1)*. The thin smears prepared from the colony for Gram staining revealed Gram-negative, pink-coloured, small rod-shaped organisms, single, paired, or arranged in short chains on microscopic examination. Thirteen *Salmonella* isolates on TSI slants showed fermentation of glucose and H2S formation, but none of the isolates fermented either lactose or sucrose. They are catalase positive, oxidase negative, indole negative, and they reproduce in citrated media (Simmon), and they do not digest urea. In Lysine-Iron Agar medium, they are purple colored "alkaline reactions".

Biochemical Identification Using the VITEK-2 System

The suspected isolates were biochemically confirmed as *Salmonella enterica* at the species level using the VITEK-2 system. Of these, twelve isolates were successfully identified, whereas one isolate could not be identified.

Real-Time PCR for Detection of *Salmonella* spp.

All thirteen suspected isolates obtained from the bacteriological examination gave positive results for the *invA* gene in real-time PCR. *Fig. 2* shows the amplification of *invA* gene in 10 *Salmonella* isolates.

Molecular Typing of *Salmonella* Serovars Using Conventional PCR

PCR amplified 261 bp DNA fragments from the *MDH* gene in all isolates, which indicates that all isolates belong to the Typhimurium serovar, with negative results obtained in other *Salmonella* serovars based on the PCR protocol and primer sets used in this study.

Table 2. *The primer sets and thermal cycling conditions of different virulence genes*				
Gene	**Primer Sequence (5'- 3')**	**Annealing Temperature (°C)**	**Amplicon Size (bp)**	**Reference**
spvB	F: CTATCAGCCCCGCACGGAGAGCAGTTTTTA R: GGAGGAGGCGGTGGCGGTGGCATCATA	66.5	717	[29]
spvC	F: ACT CCT TGC ACA ACC AAA TGC GGA R: TGT CTT CTG CAT TTC GCC ACC	58	467	[16]
spvR	F: CAG GTT CCT TCA GTA TCG CA R: TTT GGC CGG AAA TGG TCA GT	57	310	[30]
pefA	F: GCGCCGCTCAGCCGAACCAG R: GCAGCAGAAGCCCAGGAAACAGTG	66.5	157	[29]

Table 3. Salmonella isolation rate, odds ratio (OR), and P-value regarding different variables

Variable	Categories	Total Examined Animals	Number and Percentage of Positive Animals	OR (95%CI)	P-value[a]
Healthy status	Diarrheic	66	11(16.7%)	5.5 (1.16-25.97)	P=0.02
	Apparently Healthy	57	2 (3.5%)	1.0 (baseline group)	
Animal species	Cattle	62	8 (12.9%)	1.66 (0.51-5.39)	P=0. 56
	Buffaloes	61	5 (8.2%)	1.0 (baseline group)	
Age	<3 months	48	9 (18.8%)	8.77 (2.63-26.31)	P=0.046
	3-6 months	36	3 (8.3%)	3.45 (1.036-10.36)	
	>6 months	39	1 (2.6%)	1.0 (baseline group)	
Season	Summer-Spring	63	9 (14.3%)	2.33 (0.68-8.03)	P=0.242
	Autumn- Winter	60	4 (6.7%)	1.0 (baseline group)	
Sex	Female	70	8 (11.4%)	1.24 (0.38-4.03)	P=0.776
	Male	53	5 (9.4%)	1.0 (baseline group)	
Location	Faiyum	25	6 (24%)	12 (1.35-106.9)	P=0.08
	Alexandria	18	2 (11.1%)	4.75 (0.4-56.18)	
	Behera	24	3 (12.5%)	5.43 (0.53-55.52)	
	Menoufia	17	1 (5.9%)	2.37 (0.140-40.35)	
	Giza	39	1 (2.6%)	1.0 (baseline group)	

Salmonella-Associated Risk Factors Analysis

The overall *Salmonella* isolation rate from the collected fecal samples was 10.57% (13/123). Isolation was significantly higher in diarrheic animals (16.7%) than in apparently healthy animals (3.5%), with a significant association between diarrheal status and *Salmonella* isolation (P=0.02). The odds of diarrhea among *Salmonella*-positive animals were 5.5 times higher than those among *Salmonella*-negative animals. Regarding other risk factors, age had a significant effect on the *Salmonella* isolation rate (P=0.046), with animals younger than 3 months showing the highest susceptibility (OR=8.77). In contrast, no significant associations were observed between *Salmonella* isolation and other variables, including animal species, season, sex, or location (P>0.05) *(Table 3)*.

Molecular Detection of Virulence Genes in *Salmonella* Isolates Using Conventional PCR

PefA, *spvB*, *spvC*, and *spvR* were successfully amplified. Of 13 *S.* Typhimurium isolates, 8 isolates (61.5%) were positive for *spvC*, 7 (53.8%) were positive for both *spvB* and *pefA*, and 5 (38.5%) for *spvR*. Six isolates from cattle and 3 isolates from buffaloes were tested positive for different virulence genes, while 4 isolates (2 from cattle, 2

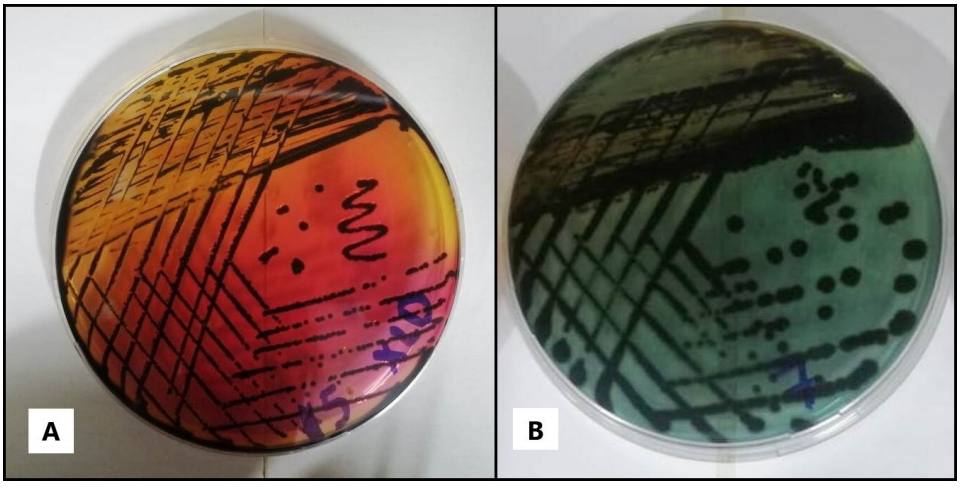

Fig 1. Typical colonies of *Salmonella* grown on selective media: **(A)** On XLD agar, colonies exhibit a black centre with a lightly transparent reddish zone, **(B)** On HE agar, colonies appear as transparent green or blue-green with black centres, sometimes presenting as almost completely black colonies

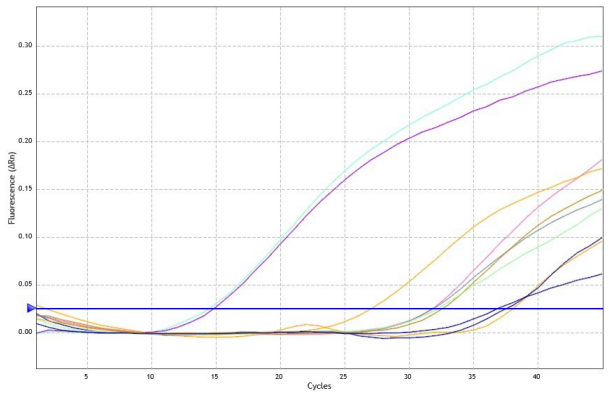

Fig 2. Shows the amplification plot of the amplified invA gene of 10 *Salmonella* isolates, which gave positive Ct after cycle 15 in 2 isolates, and the remaining 8 isolates gave positive after 25 cycles

Isolate Number	Animal species	Diarrheic	*PefA*	*SpvR*	*SpvB*	*SpvC*
1	Buffalo	✓	✓	✓	×	✓
2	Cattle	✓	✓	✓	✓	✓
3	Cattle	✓	✓	✓	✓	✓
4	Cattle	✓	✓	×	×	×
5	Cattle	✓	×	×	✓	✓
6	Cattle	✓	✓	×	✓	✓
7	Buffalo	✓	✓	×	✓	✓
8	Cattle	✓	✓	✓	✓	✓
9	Buffalo	✓	×	✓	✓	✓
10	Cattle	✓	×	×	×	×
11	Cattle	✓	×	×	×	×
12	Buffalo	×	×	×	×	×
13	Buffalo	×	×	×	×	×

Fig 3. Distribution of virulence genes among S. Typhimurium isolates recovered from Cattle and Buffaloes. (✓) indicates presence of the gene, while (×) indicates absence

from buffaloes) were negative for the four virulence genes, as shown in *Fig. 3*.

Discussion

Salmonella-induced diarrhea is a major cause of neonatal calf morbidity and mortality in several countries of the world, including Egypt, leading to significant economic losses for the beef and dairy industries [31].

In the present study, a total of 123 fecal samples were collected from cattle and buffaloes from different governorates in Egypt. Bacteriological examinations revealed 13 *Salmonella* isolates; however, the VITEK identification system detected only 12. The qPCR was used for confirmation of the results, which detected that 13 isolates (10.57%) were positive for *Salmonella*. There was an excellent correlation between the culture and PCR methods, and the PCR method did not report any false-negative results. Gebeyehu et al.[32] isolated *Salmonella* from 10.42% of the total samples, which corresponds to our findings. *Salmonella* incidence ranges from 1% as reported by Abd El-Tawab et al.[33] to 43.53% as reported by Moussa et al.[34]. These variations may be explained by differences in study location, climate, management practices, production categories used (dairy, beef, mixed), sample sources (slaughterhouse, farms, marketplace), sample type, species, age, and study protocol [35-37].

The detection of *Salmonella* by PCR results in a faster method than standard culture methods [38]. A real-time PCR *Salmonella* screening method detects *S. enterica* accurately and efficiently. It saves time, producing results in 18 to 24 h, making it an ideal method of diagnosis [39], whereas O'Hara and Miller [40] reported that the VITEK-2 system demonstrated an accuracy of 93.0% for the identification of enteric bacterial strains, as it misidentified 3 out of 15 *Salmonella* spp. Also, Simgamsetty et al.[24] detected that the VITEK-2 Compact system identified various Gram-negative bacterial strains with a level of 95-99% probability.

Following confirmation of the isolates as *Salmonella enterica*, molecular typing was performed using serovar-specific PCR assays targeting characteristic genetic markers to identify *S.* Typhimurium, *S.* Enteritidis, and *S.* Dublin. This approach was selected because PCR-based serovar identification provides rapid, sensitive, and reliable discrimination of the most epidemiologically and clinically significant *Salmonella* serovars while avoiding the technical limitations and high cost associated with conventional Kauffmann-White serotyping [28,41]. All of the *Salmonella* found in this investigation are serovar Typhimurium, which is still the most predominant serovar causing enteritis in cattle and buffalo calves, as noted in previous studies [4,34,37]. When digestive disease or diarrhea is the reason for consultation or the primary clinical symptom displayed by calves, *S.* Typhimurium is the most likely serotype involved, particularly in younger calves. Possibly, the adaptations of the neonate digestive tract, as well as the action of this serotype, favor this presentation [5].

This study assessed the impact of some risk factors, including sex, age, season, geographical location, health status, and species, on *Salmonella* detection.

This study found a statistically significant difference in the total prevalence of *Salmonella* between age groups (P=0.046), aligning with the findings of Yirsa and Tigistu [42]. The highest isolation rate was observed in animals under 3 months of age. Some precipitating factors, such as transportation, concomitant disease, or acute food deprivation, may cause disease in young animals more frequently than in adults [43].

The observed decline in fecal shedding of *Salmonella* spp. with increasing calf age suggests that younger calves are

more susceptible to intestinal colonization and subsequent bacterial shedding. This age-related pattern is biologically plausible and may reflect the progressive maturation of the immune system together with gastrointestinal development. As calves mature, rumen function becomes established, the intestinal microbiota becomes more diverse and stable, abomasal pH decreases, and the resident enteric microflora becomes more effective at limiting *Salmonella* colonization than the immature neonatal microbiota [44]. These physiological and microbial changes likely contribute to the lower prevalence of fecal shedding observed in older calves.

The prevalence was significantly higher in diarrheic animals than in apparently healthy animals, which is consistent with a previous study [22]. There are two isolates from apparently healthy animals, implying that these animals may act as a potential source of *Salmonella* infection to humans and other animals through contact. In this regard, it is worth noting that the ability of carrier animals to contaminate the environment by shedding *Salmonella* increases when animals are exposed to stressors such as transportation or holding before slaughter, or late pregnancy [43].

Although *Salmonella* frequency was numerically higher in the summer and spring, this difference was not statistically significant, indicating that season alone may not be a strong determinant of fecal shedding in this population - a pattern consistent with findings by Huston et al.[45]. This lack of a clear seasonal effect is plausibly explained by stress-related factors such as transportation, weaning, overcrowding, and concurrent disease, all of which are known to promote *Salmonella* shedding independently of season [45]. Because these stressors can occur at any time of year, they may sustain a baseline level of infection that masks or overrides any true seasonal signal. Climatic factors likely reinforce this pattern in the present setting: in tropical and subtropical regions, temperature and humidity remain within ranges favorable for *Salmonella* survival throughout the year, so the organism's environmental persistence is unlikely to fluctuate sharply with season [46]. Taken together, these factors suggest that management-related stressors and a consistently permissive climate, rather than seasonality itself, are the more relevant drivers of *Salmonella* shedding in this context.

However, the isolation rate was higher in cattle (12.9%) than in buffaloes (8.2%). No statistically significant difference was detected between the two species. Consistent with our findings, other studies [36,47] reported similar or non-significant differences in isolation rates between cattle and buffaloes. In contrast, Borriello et al.[4] demonstrated a higher susceptibility of buffaloes to salmonellosis compared to cattle. The authors attributed this increased susceptibility to differences in husbandry practices, particularly the relatively lower standards of hygiene commonly associated with buffalo rearing systems. Such conditions may facilitate the persistence and transmission of gastroenteric pathogens, thereby increasing the risk of infection in buffalo populations.

In this study, no statistically significant association was found between *Salmonella* isolation rates and geographic location. This finding is consistent with a previous study [48], which also reported that location has no significant effect on *Salmonella* detection. The lack of association in the present study may be attributed to the uneven distribution of sample sizes across the investigated localities. However, the relatively higher isolation rate observed in Faiyum governorate could be explained by the predominant use of recycled water for animal husbandry, which is more susceptible to contamination and may create favorable conditions for bacterial growth and persistence [49,50].

In this investigation, the higher prevalence of *Salmonella* isolates was observed in female cattle and buffaloes (11.4%) than in males (9.4%), although the association between sexes was not statistically significant (P>0.05). Matchawe et al.[48], Hailu and Kebede [51] also found no statistically significant difference in *Salmonella* detection between the sexes in cattle and sheep, respectively. This finding may be attributed to the fact that both males and females are generally exposed to similar environmental and management conditions, including shared housing, feed, and water supplies, which are the main routes of transmission. Although sex-related hormonal differences can influence immunological responses, they appear to have a minor impact when compared to stronger risk factors, including age, hygiene, stocking density, and stress. As a result, the role of sex as a determinant of *Salmonella* infection is probably obscured by these more influential factors, resulting in the lack of a significant association [48].

The virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors [52,53]. In this investigation, all isolates carried the *invA* gene, which was expected since *invA* is an invasion gene conserved among *Salmonella* serotypes.

PCR amplification of virulence genes *pefA, spvB, spvC,* and *spvR* was performed using plasmid DNA because these virulence genes are encoded on the *Salmonella* virulence plasmid. Accordingly, plasmid DNA was considered an appropriate template for the specific detection of these plasmid-associated virulence genes [54,55].

The results revealed that different virulence genes were found in the *Salmonella* isolates under investigation. Additionally, the study's findings support the idea that there is genetic differentiation between isolates of the same serotype in the distribution of virulent genes, as in

a previous study [56], since both *spvB* and *pefA* genes were detected in 53.8% of the isolates, *spvC* was detected in 61.5% of the isolates, while the *spvR* gene was detected in 38.5% of the isolates. Chuanchuen et al.[57] found the virulence plasmid-associated genes *spvC* and *pefA* in only two (1.3%) and one (0.6%) *Salmonella* isolates obtained from dairy cows, respectively. Also, Dahshan et al.[52] found that out of 17 *S.* Typhimurium isolates, 14 isolates were positive for the *spvC* gene, and 13 were positive for the *pefA* gene. Amini et al.[58] detected *spvB* and *spvC* genes in 100% (15/15) of *S.* Typhimurium from bovines. Sudrik et al.[59] found *spvR* in 26.31% of *Salmonella* isolated from cattle farms.

The *pefA* gene was associated with the Typhimurium serotype; the association between the Typhimurium serotype and the *pefA* gene may explain the high frequency of diarrhea in the presence of this serotype, as this gene allows bacterial adhesion to intestinal epithelial cells [5].

However, variations in the detection of *Salmonella* virulence genes, particularly *spvB*, *spvC*, and *spvR*, have been reported across different studies. Experimental and epidemiological evidence from both animals and humans suggests that these genes are not essential for the development of gastroenteritis or diarrhea caused by various *Salmonella* serovars, including *Salmonella enterica* serovar Typhimurium. Instead, their role appears to be more critical in cases of extraintestinal infections [18,60]. Nevertheless, some studies indicate that *Salmonella* isolates harboring virulence plasmids may be associated with more severe forms of diarrhea [60].

In conclusion, *Salmonella* is prevalent in cattle and buffaloes in Egypt. Quantitative PCR (qPCR) demonstrates higher detection performance for *Salmonella* isolates than the VITEK-2 system in the present study. *Salmonella enterica* serovar Typhimurium was identified as the only detected serovar based on the PCR protocol and primer sets used in this study. The isolation rate is significantly higher in diarrheic animals compared to apparently healthy ones. Age is significantly associated with *Salmonella* isolation, with animals younger than 3 months being the most susceptible, whereas species, sex, season, and geographic location show no significant effect. Plasmid-associated virulence genes *(spvB, spvC, spvR,* and *pefA)* are detected at varying frequencies among the isolates, suggesting a limited role in the pathogenesis of diarrhea.

Declarations

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