Prevalence and Molecular Characterization of Salmonella Serovars Isolated from Diarrheic Cattle and Buffalo-Calves

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Abstract

In the first 10 weeks of life, bovine salmonellosis is the most serious infection typically affects calves. The aim of this work was to study the prevalence, antimicrobial susceptibility profile, attributes of some virulence and resistance genes of Salmonella isolated from diarrheic cow and buffalo-calves. A total of 200 fecal samples from cow and buffalo-calves were bacteriologically examined for isolation of Salmonella species. The percent of positive cases (n= 65 /200) was 32.5%. Serological typing of the recovered Salmonella isolates produced eight serotypes, Salmonella Typhmurium (13.8%), S. Anatum (7.6%), S. Sankjohann (1.5%), S. Salami (20%), S. Mississippi (24.6%), S. Stratford (13.8%), S. Enteritidis (7.6%) and S. Saintpaul (10.7%). Upon ower knowledge, this is the first record of isolation of S. Sankjohann from diarrheic calves in Egypt. The results revealed a higher incidence of salmonellosis in Spring (57.6%) followed by Winter (27.9%). Also, the incidence of salmonellosis was more recorded in cow calves (43.58%) than buffalo calves (16.86%). Antimicrobial susceptibility testing showed that the highest sensitivity levels were found for nalidixic acid (75%), enrofloxacin (62.5%), and chloramphenicol (50%) whereas, all isolates (100%) were resistant to ampicillin, gentamicin, streptomycin, and doxycycline. The 4 virulence genes (invA, avrA, stn, spvC) were found in the 8 examined Salmonella isolates. The blaTEM and tetA(A) resistance gene were detected in all isolates that were resistant to ampicillin and doxycycline. Tetracycline resistance gene (floR) was identified in 5 isolates; the sul1 gene was present in Sulphamethoxazole resistant isolates and the dfrA gene was present only in 2 isolates (S. Sankjohan and S. Mississippi) which existed resistance to trimethoprim. By comparing the stn gene sequence data of both S. Sankjohann and S. Stratford with other Salmonella strains from the GeneBank the point mutation (Threonine 371 to Serine) was identified. In conclusion, this study proved the presence of different virulent and MDR salmonella isolates in diarrheic calves that make persistence shedding of microorganism into the environment. Moreover, antimicrobial sensitivity testing should be performed prior to treatment of Salmonella infection.

Keywords: Bovine salmonellosis, Calves, Salmonella serotypes, Antimicrobial resistance, Virulence genes.

Introduction

Salmonellosis is a major endemic disease of calves, which has been reported by an increase in incidence, in particular that caused by Salmonella Typhimurium in calves of intensive rearing systems [1]. Salmonellosis is a zoonotic disease that can cause serious infection in both calves and adult cattle. Clinical symptoms of bovine salmonellosis may include diarrhea fever, anorexia, dehydration, abortion, and endotoxemia evidence though many infections remain subclinical [2]. The monitoring of drug resistance patterns among Salmonella isolates not only gives vital clues to the clinician on the best therapeutic regime in each individual case, but is also an important tool in devising a comprehensive chemoprophylactic and chemotherapeutic drug schedule within a geographical area [3]. PCR is important tool to...
develop a highly sensitive and precise diagnostic method for rapid detection of Salmonella species in calves [4]. The invasion A (invA) is one of the most studied virulence factors that is also used as a biomarker for Salmonella spp. detection as it contains sequences that are unique to the genus Salmonella [5]. The spvC is virulence-related gene on the plasmid required for survival within host cell [6]. AvrA gene is an effector protein of the type III secretion system (TTSS) complex that contributes to the virulence of Salmonella spp. by limiting the host’s inflammatory responses through the inducement of cell apoptosis, especially of macrophages, and by the inhibition of IL-8 and TNF-α [7]. Salmonella enterotoxin gene that encoded stn induces more loss of intestinal fluids causing diarrhea [8]. Resistance to β-lactam antimicrobial agents in E. coli is primarily mediated by β-lactamases, which hydrolyze the β- lactam ring and thus inactivate the antibiotics [9]. At least nine different florfenicol resistance genes have been identified including floR [10]. Resistance to tetracycline is governed by tet genes, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification [11]. Thus, in this study we investigated the prevalence rate of salmonellosis among newly born cow and buffalo calves. In addition, the phenotypic resistance pattern of the recovered isolates, some antimicrobial resistance genes and virulence genes were determined.

Materials and Methods

Animals and clinical samples

Over the period from December 2018 to October 2019, a total of 200 diarrheal calves (117 cows and 83 buffalo) from large farm animals and sporadic cases from three governorates (Gharbia, Menoufia, Qaliubiya) attended the veterinary clinic. Rectal swabs were collected from diarrheal calves for bacteriological examination. All samples have been sent to the laboratory in an ice box, with minimal delay for bacteriological testing.

Bacteriological isolation

The rectal swabs were inoculated into tubes contain buffered peptone water for pre enrichment then each culture of pre-enrichment has been inoculated into Selenite F broth (Oxoid, UK) then each enrichment culture was streaked on a selective agar as into the xylose lysine deoxycholate (XLD, Oxoid, UK) agar for the isolation of Salmonella [12]. Suspected colonies were tested biochemically (urease production, methyl red (MR) and voges-proskauer (VP) tests, lysine decarboxylase production, citrate utilization, H2S production and indole production) as documented previously [13].

Serological identification

Serological typing of Salmonella isolates was done using the modified Kauffman-White scheme as documented previously [14].

Antibiotic susceptibility testing

All the recovered Salmonella isolates (n=8) from diarrheal calves have been tested against 10 antimicrobial disks (Oxoid, UK) for their antimicrobial sensitivity using the standard disc diffusion methods [15]. The tested antimicrobials included Gentamicin (10 μg), Streptomycin (10 μg), Doxycycline (30 μg), Norfloxacin (10 μg), Enrofloxacin (5 μg), Nalidixic acid (30 μg), Ampicillin (10 μg), Ampicillin (10 μg), Levofloxacin (5 μg), Chloramphenicol (30 μg), and Trimethoprim-sulphamethoxazole (1.25 + 23.75 μg). The results were interpreted according to Clinical Laboratory Standard Institute (CLSI) guidelines [16].

Molecular characterization of some virulence and resistance genes

The recovered salmonella isolates were tested for 4 virulence (invA, Stn, avrA and spvC) and 5 antibiotic resistance (blaTEM, floR, sul1, tetA(A), and dfrA) genes (Table 1). The bacterial DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s guidelines. The PCR reaction mixture consisted of 12.5 μL of Emerald Amp GT PCR master mix (Takara),1 μL of each set of forward and reverse primers (20 pmol), (Eurofins Pvt. Ltd., Bangaluru), 5 μL of DNA as a template and nuclease free water to make 25 μL of reaction volume. The PCR cycling conditions were programmed according to the reference of the primer (Table 1). The amplified PCR products were resolved by agarose gel electrophoresis, using 1.5% agarose gel stained with ethidium bromide (0.5 μg/mL) and visualized and documented using UV gel documentation system (Alpha Innotech, Biometra).
Table 1: Oligonucleotide primer sequences used in investigation of *Salmonella* species isolated from diarrheic calves

<table>
<thead>
<tr>
<th>Primer use and target gene</th>
<th>Sequence (5’→3’)</th>
<th>Amplified product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resistance genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bla</em>TEM</td>
<td>ATCAGCAATAAAAACCAGC</td>
<td>516</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>CCCCGAAGGAAGTTTTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>floR</em></td>
<td>TTGGWGGCCTMTCRGAC</td>
<td>494</td>
<td>[18]</td>
</tr>
<tr>
<td><em>Sul1</em></td>
<td>SGAGARAAGACGAAGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGGCCGGTGGCTACCTGAAGC</td>
<td>433</td>
<td>[19]</td>
</tr>
<tr>
<td><em>tetA(A)</em></td>
<td>GCCGATCGCGTGAAATGGCC</td>
<td>576</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTGTCCGACAAGTTCGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Virulence genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Stn</em></td>
<td>TGGTGTCGCATACCAGGAATGGAGT</td>
<td>425</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>TATGTTAGGGCAAGTTCGATGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>invA</em></td>
<td>GTGAAATTATCGCCACGTTCGGCA</td>
<td>284</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>TCCACCGTCCGATGGAAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>avrA</em></td>
<td>CCTGTAATGGACGTCTGCGG</td>
<td>422</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>AGAAGAAGATGGTCGTAATGCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>spvC</em></td>
<td>ACCAGAGATGGCCCTCTGCGG</td>
<td>467</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTGTAGCCAGCTGATAATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNA sequencing and phylogenetic analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The obtained PCR products of two isolates were purified by Qiaquick PCR purification kit (Qiagen Inc. Valencia CA) according to the manufacturer’s Guidelines. Sequence analysis was performed to determine nucleotide composition of the strain detected for genotypic analysis and this was applied in both directions using the previously mentioned forward primer and reverse primers of *stn* gene by 3730 DNA Analyzer, Applied Biosystems, USA. Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, UK). The manufacturer's protocol had been used as recommended. Sequences alignment (224 bp fragments of VP1) and Creating phylogenetic tree (Neighbor-joining) to detect genetic similarity of the strain tested of the current study compared to other strains worldwide registered in GeneBank were carried out using BioEdite software program V.5.0.9 [24] and MEGA-7 software program [25]. The identified strains were *S. Stratford_SH_QS1* with accession NO. MT019960 and *S. enterica_SH_AS2* (S. Sanktjohann) with accession NO. MT019961.

**Results**

**Prevalence of Salmonella serotypes among diarrheic calves**

The clinical examination of 200 diarrheic calves revealed variable consistency of diarrhea (watery, pasty, mucoid and bloody), fever, with different grades of dehydration, and paleness in mucous membrane. Some animals suffered from respiratory manifestation.

Out of 200 bacteriologically examined rectal swabs, 65 samples were positive. *Salmonella* isolates on XLD media were pink with black center and categorized to 8 serogroups. All isolates were positive for catalase, methyl red, and lysine decarboxylase tests and negative for indole, VP, oxidase test and urea hydrolysis. On TSI agar *Salmonella* not ferment lactose and produced red slant and yellow butt with H2S production. All *Salmonella* isolates were motile on semisolid agar media. The rate of *Salmonella* infection was 16.86% (14/83) and 43.58% (51/117) among the examined diarrheic buffalo and cow calves, respectively. The prevalence rates in Spring were 72.4% and 39.13% and in Winter were 40.42% and 2.8%, while in Summer
were 32.35% and 0% in cow and buffalo calves, respectively. The identified serotypes were S. Typhmurium and S. Anatum from the examined diarrheic buffalo calves, S. Salami, S. Mississippi, S. Sanktjohann, S. Stratford, S. Saintpaul, and S. Enteritidis from cow calves (Table 2).

Table 2: Serotypes, resistance phenotype, virulence and resistance genes of the isolated Salmonella from the examined calves

<table>
<thead>
<tr>
<th>Specie</th>
<th>Age (days)</th>
<th>Sex</th>
<th>Locality</th>
<th>Clinical signs</th>
<th>Serovar</th>
<th>Resistance profile</th>
<th>Virulence genes</th>
<th>Antimicrobial resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo calf</td>
<td>120</td>
<td>Male</td>
<td>Gharbia</td>
<td>Watery diarrhea, weakness, anorexia, fever</td>
<td>Typhmurium</td>
<td>Am, CN, S, DO, ENR, SXT and C</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A) and Sul1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Female</td>
<td>Gharbia</td>
<td>mucoid diarrhea with fetid odor, increase temp (39.9 °C)</td>
<td>Anatum</td>
<td>Am, CN, S, DO and SXT</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A) and Sul1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Female</td>
<td>Monofia</td>
<td>Profuse watery diarrhea, subnormal temperature (36 °C) dehydration</td>
<td>Salami</td>
<td>Am, CN, S, DO, SXT and C</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and dfrA</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>Female</td>
<td>Monofia</td>
<td>mucoid diarrhea with soiled tail, offensive odor, normal temp (39.2 °C)</td>
<td>Mississippi</td>
<td>Am, CN, S, DO, LEV, NOR, ENR, SXT, C and NA</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and dfrA</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Female</td>
<td>Monofia</td>
<td>mucoid diarrhea, fever (41 °C), red m.m</td>
<td>Sanktjohann</td>
<td>Am, CN, S, DO, LEV, NOR, ENR, SXT, C and NA</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and floR</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>Female</td>
<td>Monofia</td>
<td>Pasty diarrhea, normal temperature (39.2 °C)</td>
<td>Stratford</td>
<td>Am, CN, S, DO, NOR and SXT</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and floR</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Female</td>
<td>Qulubia</td>
<td>mucoid diarrhea, fever (41.7 °C) high respiratory rate, nasal discharge</td>
<td>Saintpaul</td>
<td>Am, CN, S, DO, LEV, NOR and SXT</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and floR</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>Male</td>
<td>Qulubia</td>
<td>watery diarrhea, normal body temp (38.9 °C)</td>
<td>Enteritidis</td>
<td>Am, CN, S, DO, NOR and SXT</td>
<td>Stn, invA, spvc and avrA</td>
<td>blaTEM, TetA(A), Sul1 and floR</td>
</tr>
</tbody>
</table>

Resistance phenotypes of the recovered Salmonella isolates

All tested Salmonella isolates (100%) were resistant to ampicillin, gentamicin, streptomycin, doxycycline and sulphamethaxazole + trimethoprim. Meanwhile, 62.5% of the isolates were resistant to norfloxacin, and 50% to chloramphenicol. The lowest resistance rate was observed against levofloxacin and enrofloxacin (37.5%) and nalidixic acid (25%).

Virulence characteristics and determinants of antibiotic resistance

PCR technique was used as a molecular tool in this study to detect 4 virulence genes (invA, avrA, stn, spvC). It was found that, all 4 virulences genes were detected in all the 8 salmonella isolates with percentage of 100% (Figure 1). All isolates which were resistant to doxycycline, ampicillin, and sulphamethaxazole were positive for tetA(A), blaTEM, and sul1 genes (Figure 2). Two isolates which existed resistance to trimethoprim yielded 425bp amplicons for dfrA gene and 5 tetracycline resistant isolates produced 494 bp amplicons for floR gene (Table 2).

PCR successfully amplified the stn gene with band of amplification size at 617 bp from the isolates. After sequencing and analysis of the 617 bp PCR products of S. Sanktjohann and S. Santipaul with the other Salmonella strains on the GeneBank database, the point mutation (Threonine 371 to Serine) was identified. The phylogenetic analysis indicated that S. Sanktjohann that belongs to S. Enterica (GeneBank accession NO. MT019961) has identity percent of 99.7% with S. Sloterdijk ATCC15791 (CP012349), S. Paratyphi A ATCC9150 (CP000026), and S. Paratyphi A ATCC11511 (CP019185). The identified S.Stratford (MT019960) has 100% identity with S. Typhimurium ATCC 13311 (CP009102), S. Typhimurium PIR00538 (CP025555), and S. Typhimurium 01ST04081 (CP029840) (Figure 3).

![Figure 1: Agarose gel electrophoresis revealed amplification product for the avrA gene at 422bp (a), invA at 284bp (b), stn at 617 bp (c), and spvC gene at 467 bp (d). Lane L 100bp DNA molecular size marker, lanes Pos. and Neg. positive and negative controls, respectively. Lanes (1-8) referred to the examined Salmonella isolates from diarrheic calves.](image-url)
Figure 2: Agarose gel electrophoresis revealed amplification product for the tetA(A) gene at 576 bp (a), sul1 at 433 bp (b), dftrA at 425 bp (c), floR at 494 bp (d) and blaTEM antibiotic resistant gene at 516 bp (e). Lane L 100bp DNA molecular size marker, lanes Pos. and Neg. positive and negative controls, respectively. Lanes (1-8) referred to the examined Salmonella isolates from diarrheic calves.

Figure 3: Phylogenetic tree of stn virulence gene showing the genetic relationship of Salmonella S. Sanktjohann (accession NO. MT019961) and S. Stratford (MT019960) isolated from diarrheic calves and the other Salmonella spp. available from the GeneBank.
Discussion

Salmonellosis is a common disease of bovine and calves [26]. This study was planned to identify the prevalence of *Salmonella* among cases of cattle diarrhea with special reference to some of their virulence genes and antimicrobial sensitivity profile. Upon clinical examination of diarrheic calves, the diarrhea was graded in 4 classes according to the consistency of the fecal matter and it was found that, the majority of cases suffered from mucoid diarrhea and increase in body temperature and this findings was as before mentioned [27] that calves infected with salmonellosis showing clinical symptoms include fever, sluggish mentation, loss of appetite and scores that often include increased mucus and blood. In the present study, *Salmonella* Enteritidis was isolated from a male cattle calf at one month of age suffering from watery diarrhea, normal body temperature (38.9°C) with dehydration. It was also isolated from diarrheic calves [28, 29]. As previous report [30], *S.* Saintpaul was recovered from a male at one-month of age exhibiting mucoid diarrhea, fever (41.7°C), rapid respiratory rate, nasal discharge and congested mucous membrane.

Unlike some other studies [31] the highest seasonal rate of salmonellosis among the examined diarrheic cow and buffalo calves were in spring season followed by winter and summer. These variations may be due to the exposure to stressors in winter and spring seasons such as transport, starvation, overcrowding and change in temperature. Out of 200 samples, 65 (32.5%) was positive for salmonellosis, 14 swabs from buffalo calves and 51 swabs from cow calves. This percent is higher than previous study (10.7%, 21/195) [32].

The antimicrobial resistance of *Salmonella* species associated with horizontal transmission of antibiotic-resistant genes among *Salmonella* strains and other *Enterobacteriaceae* or clonal spread of antimicrobial drug-resistant serovars that are successful in worldwide dissemination [33]. The *invA* gene was present in all isolates as detected 284 bp PCR amplicon [34]. The *avrA* is an SPI-1 effector protein involved in the enteritis pathway, with critical roles in inhibiting inflammation and apoptosis and *AvrA* is secreted by both type three secretion system (T3SS)-1 and T3SS-2 [35]. In our study, *AvrA* gene was present in all recovered 8 *Salmonella* isolates, our findings are in accordance with previous results [35] that *avrA* gene was present in 100% of the isolates. The *sin* gene was detected in all tested isolates (617 bp). This result differs from previously mentioned that *sin* gene was detected only in 20% of isolates [36]. Also, the *spvC* gene was detected in all tested *Salmonella* strains (100%) at a 467 bp which is in agreement with Giacomodonato et al. [35], who found that *spvC* gene was present in 92% of tested isolates. Moreover, the *tetA(A)* gene was detected in all 8 recovered *Salmonella* strains at (576 bp) which showing resistance to doxycycline that result was not as mentioned previously [4].

There are more records of antibiotic resistance and multiple drug-resistant salmonellosis in developing countries as 31.8% of *Salmonella* isolates in sheep and goats, 44.4% in camel isolates, and 52% in bovine isolates were resistant to the widely used antimicrobials [37]. In this study all *Salmonella* isolates were resistance to ampicillin as mentioned before [4]. However, another study [38] found the resistance to ampicillin was 58%. The result of antimicrobial sensitivity test of *Salmonella* isolates against aminoglycosides group showed that gentamycin and streptomycin not had any sensitivity against all *Salmonella* isolates (resistant rate 100%). Nevertheless, previous study [39] declared that 53.2% of *Salmonella* isolates were multidrug resistant (MDR) and 76.9% were resistant to streptomycin while the majority of the isolates were susceptible to gentamycin. In addition, Abd El-Rahman et al. [38] detected that the highest sensitivity was observed for streptomycin (80%) and gentamycin (75%).

All *Salmonella* isolates were resistant to doxycycline. This result was in contrast to that reported by Abd El-Rahman et al. [38] who found that the resistance rate against tetracycline was 67%. However, Atyabi [40]
revealed that all *salmonella* isolates were resistant to doxycycline and erythromycin. In contrast to previous findings [4], 75% of the isolates were susceptible to nalidixic acid.

Sulfonamides make their action through interfering with the synthesis of folic acid of microorganisms by competing with *P*-aminobenzoic acid (PAPA) in the biosynthesis of Dihydrofolate [41]. Our results showed that, the sensitivity of the isolates to sulphamethoxazole/trimethoprim was 0% and these results was completely agreed with Shekhar and Singh [42] who found that the maximum resistance was observed against sulphamethoxazole was 100%. Although 50% of the tested isolates were sensitive to chloramphenicol, Shekhar and Singh [42] reported that the highest level of sensitivity among salmonella isolates was to chloramphenicol (100%).

The percent of tetA (A) gene was 83.7% in all tested *Salmonella* isolates. However, Adesiji *et al.* [43] detected tetA (A) gene at a percent of 100%. Sul genes are those genes responsible for conferring resistance to sulfonamide drugs. Similar to previous findings [4], the sul1 gene was detected at a 433 bp in all tested *Salmonella* strains.

**Conclusion**

The obtained results proved the detection of virulent and multidrug resistant *Salmonella* serotypes from diarrheic calves. Therefore, the use of specific antimicrobial drug for treating *Salmonella* infection after application of sensitivity test is still a must.

**Conflict of Interest**

The authors have no conflict of interest to declare.

**Acknowledgment**

The authors would like to thank Veterinary Serum and Vaccine Research Institute, Egypt for covering all the expenses of the experiment needed to conduct the work.

**References**


Canberra: Food Standards Australia New Zealand (FSANZ).


