ISOLATION AND MOLECULAR IDENTIFICATION OF THE invA GENE OF Salmonella spp. IN DROMEDARY CAMELS

Ali Kadhim ALTAEE and Afaf Abdulrahman YOUSIF

Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, P.O. Box 28601 AdDawoodi, Iraq

Email: afaf.a@covm.uobaghdad.edu.iq

ABSTRACT: This study was done to determine the percentage of Salmonella spp. in camels from three provinces (Karbala, Al-Nafaj and Al-Muthana) in Iraq with different age and both sexes. Total of 250 fecal samples from 250 camels were collected. Diagnostic study depended upon the morphological and cultural properties of the isolates on some selective media like Xylose lysine deoxycholate (XLD) and Salmonella Shigella (SS) agars which were used in addition to different biochemical tests and molecular assay by PCR for detection of virulence gene invasion A (invA) with Phylogenetic study. The clinical signs appearing on animals infected with Salmonella were greenish diarrhea, loss of appetite with mild systemic reaction. Bacteriological and molecular tests revealed isolation of five Salmonella isolates with invA gene. Two of these isolates were sequenced. The results showed that the first strain S. enterica subspecies typhimurium (LC730846) converged with a group of global strains with one node, as it converged with the global strain that held the clade (MK017934.1 and MT460418.1). While the second local strain S. enterica serovar enteritidis (LC730849) appeared with a new node and it is not affiliated with any association with the world S. enterica strains. It is concluded that the presence of Salmonella spp. in camels needs monitoring in order to minimize the risks of infection exposing human beings.

Keywords: Camels, Fecal samples, invA gene, PCR, Salmonella.

INTRODUCTION

Salmonellosis is an important infectious zoonotic disease that affects the health and economic effect of industrialized and developing countries. In developing nations, salmonellosis is often a common but neglected disease (Mohammadpour et al., 2020). Members of genus Salmonella are ubiquitous in nature. They live in the human (Abebe et al., 2020), various animals (Afaf et al., 2010; Al Zubuidy and Yousif, 2012), animals such as goats, dogs and cows (Afaf et al., 2010; Al Zubuidy and Yousif, 2012) respectively and Pets contaminate the environment of their owners by shedding Salmonella intermittently in their feces (Dróżdż et al., 2021).

Salmonellosis is characterized by enterohaemorrhagic or enteric manifestations, which result in several clinical signs, that include neonatal diarrhea, abortion, orchitis, pneumonia, and septicemia (Abdelwahab et al., 2019). Chronic salmonellosis in camels is characterized by diarrhea, weight loss and death within a few weeks, Humans can be infected by the consumption of contaminated foods originated from camels, infected drinking water, or close contact with infected camels (Wernery and Kaaden, 2002).

Salmonella infection of camels is reported in Sudan, UAE, Palestine, France, Somalia, North Africa, USA, Ethiopia, Egypt, and Iran (Sepehr, 2012). Sevilla-Navarro et al. (2021) reported that the prevalence of Salmonella in camels was 5.5% (3/54), and the only serovar isolated was S. Frintrop. Using pulsed-field gel electrophoresis analysis which revealed low genetic diversity, and all isolates showing nearly identical pulsortype (similarity >95%), they indicate that dromedary camels seems to be a reservoir for Salmonella transmission especially camel riding has become as one of the main touristic attractions in different countries which increases the popularity in recent years.

The pathogenesis of salmonellosis was begins with the local colonization of bacteria, followed by bacterial spread of infection to other regions of body, which leads to different clinical signs according to the virulence of Salmonella strain (Robert et al., 2013). Twenty eight serotypes of Salmonella were isolated from camels in United Arab Emirates by Winery (1992), these serotypes were identified with the most frequent S. saintpaul, followed by S. frintrop, S. hindmarsh and Salmonella typhimurium.

Camels are an important source of Salmonella. It is, therefore, important to control and prevent salmonellosis in these animals and their products to decrease the transmission to a human. Therefore, this study aimed at isolating and identifies Salmonella species in camels with determining their virulence gene invA via conventional PCR assay with phylogenetic analysis.

MATERIAL AND METHODS

Ethical committee
This study was approved by the ethical and research committee of Veterinary Medicine of College, University of Baghdad, book No. 39/D A in 7/12/2021.

Animals and samples
The study was performed on 250 camels at field located in three provinces in Iraq [Karbala (36), AL Najaf (191) and AL Mothana (23)]. Camels aged from one day -seven years, and were from both sexes. The study extended from January 2021 to December 2021. Data and history obtained from owner, clinical examination are recording, systemic reaction (temperature, pulse, respiratory rates), and presence or absence of diarrhea and other signs (Al-Graibawi et al., 2021). 250 fecal samples were collected from these camels and put in sterile container and kept on ice till reaching the laboratory.

Isolation and identification of Salmonella
Fecal samples (1 gram) were transferred in sterile container immediately to the lab in ice box. The samples were cultivated on Selenite-F Broth and MacConkey agar and incubated aerobically at 37 °C for 24-48 hours. Microscopical examination by Gram staining was done according to Quinn et al. (2004) and the bacterial cells were examined using X100 lenses with immersion oil. After cultivating suspected colonies on Xylose lysine deoxycholate (XLD) agar and Salmonella Shigella (SS) agar was confirmed by Gram staining and different biochemical tests (oxidase, urease, indole tests, catalase, TSI and citrate utilization).

Antibiotic susceptibility test
Antibiotic susceptibility of isolates was determined by disc diffusion method (Bauer et al., 1966) on Muller Hinton agar. The test was done by using different antibiotic discs according to (Quinn et al. 2004, Arcan and Afaf, 2013). The antibiotic inhibition zone was estimated as mentioned the Clinical and Laboratory Standards Institute (CLSI, 2023).

Molecular assay: PCR and sequencing
Primers used in this study were obtained from Bioneer Company, Korea and were designed based on the sequence of the invA gene: Forward primer GTGAAATTATCGCCACGTTCGGGCAA and reverse primer TCATCGACCGTGCAAAGGAACC with an expected amplicones size of 280 bp (Cocolin et al., 1998).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer /sequence 5’ to 3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>F GTGAAATTATCGCCACGTTCGGCAA</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>R TCATCGACCGTGCAAAGGAACC</td>
<td></td>
</tr>
</tbody>
</table>

DNA extraction and PCR program
The isolation of DNA genomic from bacterial growth done according by using DNA extraction kit (Addprep bacterial genomic DNA extraction kit) from Addbio Company (Korea). The purity and concentration of the final template DNA after extraction were measured by Quantus Fluoro meter (Promega Company, Korea). Colonies suspected to be Salmonella by conventional phenotypic methods were placed in 500 µl of distilled water and boiled for 10 min to release the genomic DNA and subsequent detection of the invA gene by PCR. The strains were stored at -70°C for their future sequencing analysis. The PCR reaction cocktail was contained of 50 mM KCl, 10 Tris-HCl mM pH 9, 0.1% Triton X-100, 2 mM MgCl2, 0.01% of gelatin, 0.2 mM of each dNTP, 1 µM of invA primer (Bioneer, Korea), 1U of Taq DNA polymerase (Highway®) and 5 µl of DNA. The thermal profile used for the detection of the invA gene was detailed in (Table 1). The PCR were analyzed by electrophoresis in a 2% agarose gel in the presence of ethidium bromide. The band size was determined by comparing the products amplified with the molecular size marker DNA Ladder 100 bp (Promega®, Korea).

<table>
<thead>
<tr>
<th>Table 1 - PCR thermocycler condition of invA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermocycling</td>
</tr>
<tr>
<td>1- Initial denaturation</td>
</tr>
<tr>
<td>2- Denaturation</td>
</tr>
<tr>
<td>3- Annealing</td>
</tr>
<tr>
<td>4- Extension</td>
</tr>
<tr>
<td>5- Final extension</td>
</tr>
<tr>
<td>6- Hold</td>
</tr>
</tbody>
</table>

Sequence analysis
All samples with positive PCR product (20 µl) were sent to (Macrogen company, Korea) for sequence analysis by using sanger macrogen analyzer for determination sequence variation among the isolates, the results was analysis by using Bioedit software version (3.1) and phylogenetic tree analysis was performed by mega 11.0. Based on the NCBI-BLAST data, Multiple Sequence Alignment Analysis of invA gene, phylogenetic tree, and homology sequence identity were made with the Genbank-NCBI strain/isolate (Gharban and Yousif, 2020).
Statistical analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference--LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

RESULTS

Clinical signs

Clinical signs included diarrhea with greenish color feces (Figure 1), and with yellowish to white color (Figure 2). Other clinical signs were loss of appetite, moderate dehydration, dullness, congestion of mucous membranes and slight increase of temperature, pulse and respiration rates.

Isolation of Salmonella

From 250 fecal samples collected from diarrheic and non-diarrheic camels, 5 isolates of Salmonella (2 %) were determined. Cultivation characteristics of Salmonella spp. isolates showed colorless, smooth colonies on MacConkey agar, On Salmonella Shigella (SS) agar the organisms were produced small sized with black pin-head, circular or round smooth, raised, colorless (Figure 3A), while the colony was pale pink with large black center on Xylose Lysine (XLD) agar (Figure 3B). The colony on smears on Gram’s stain showed G-ve bacteria pink in color, the shape appeared as small rod under the microscopic. To classify the isolates of bacteria, a number of biochemical tests were conducted on Salmonella isolates as shown in (Table 2).
Table 2 - Results of Salmonella isolates on biochemical tests.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Indole</th>
<th>VP</th>
<th>Citrate</th>
<th>H₂S</th>
<th>Motility</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Antibiotic susceptibility of Salmonella spp.

The antibiotic susceptibility of Salmonella spp. is presented in Table 3 and Figure 4. The results revealed that the 5 (100%) of Salmonella spp. isolates were resistant at least 9 antibiotic; Gentamicin, Erythromycin, Tetracycline, Ciprofloxacin, Amikacin, Sulfamethoxazole, Trimethoprim, Nalidixic acid and Cefotaxime. The 5 Salmonella spp. isolates revealed susceptibility to 4 antibiotic classes.

Table 3 - Antibiotic drugs against salmonella spp. isolates. S: sensitive isolates, R: resistant isolates to antibiotic

<table>
<thead>
<tr>
<th>Antibiotic (concentration)</th>
<th>S (%)</th>
<th>R (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (10 µg)</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin (30 µg)</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Gentamicin (30 µg)</td>
<td>0 (0%)</td>
<td>12 (0%)</td>
<td></td>
</tr>
<tr>
<td>Erythromycin (60 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline (5 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (10 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Amikacin (10 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Sulfamethoxazole (30 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim (30 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>12 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid (30 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime (25 µg)</td>
<td>0 (0%)</td>
<td>12 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

P-value | 0.0074 ** | 0.0069 ** | -

**: P≤0.01

Results of molecular assay

In PCR technique, all the Salmonella isolates amplified a 248 bp DNA amplicones, which suggested the presence of invA and further confirmed that all the isolates were Salmonella.

Identification of clades in phylogenetic tree

Present results obtained two serotypes of Salmonella namely Salmonella enteritidis and Salmonella typhimurium by sequencing analysis with two accession number from gene bank websites as (LC730849, LC730846) respectively (Figure 6).
DISCUSSION

There are various studies showing the prevalence and seroprevalence of *Salmonella* spp. in camel farms, as well as the risk factors associated with the presence of the bacterium and the prevalence in slaughter house (Wernery, 1992; Bosilevac et al., 2015; Sevilla-Navarro et al. 2021).

The criteria for the cultural and biochemical identification of *Salmonella* spp. are widely described and the detection of *Salmonella* spp. by conventional culture is considered the reference method (AL-Darraji and Yousif, 2012). However, this method suffers from the disadvantage of the time required for the obtaining a result, these tests depend on the appearance of the analyzed characteristics and can be affected by variations in culture media and in the incubation conditions. Alternatively molecular methods are being used more and more; allowing a faster and simpler diagnosis (Tracogna et al., 2013).

Currently, *Salmonella* is detected by standard bacteriological, biochemical and serological techniques. These techniques are generally time-consuming, tedious and expensive as they require hundreds of antisera as well as well-trained technicians (Nora and Thong, 2010). Many researchers underlined the importance and necessity of founding a more rapid and effective detection methods as a basis of controlling the infection (Al-Zubaidy et al., 2015). Several rapid and sensitive methods have been developed for identification of *Salmonella* serotypes from clinical specimens (Salih and Yousif, 2018a).

All strain of *Salmonella* isolates were resistance to many antimicrobial agents, the findings of the current study were in accordance with Kipper et al. (2019) who found (100%) *Salmonella enterica* isolate carrying this region of resistance in the petri dish among 63 isolates. Lekagul et al., (2020) mentioned that the *Salmonella* spp. have the ability of spread the antibiotic resistance by the genes called transfer-associated, thus, so causing increase in the incidence and the severity of the disease. Also the treatment of salmonella in all animals was difficult due to multidrug-resistant of *Salmonella* spp.

*Salmonella* specific PCR with primers for invA is rapid, sensitive and specific for detection of *Salmonella* in many clinical samples. The detection of a 284 bp product of the invA gene, specific for *Salmonella* spp., was achieved by using the conventional PCR variant. This PCR technique has been shown to be reproducible, specific and sensitive for the detection of *Salmonella* spp. It is recommended that the standardization of a PCR be carried out in each laboratory. The

**Figure 6** - phylogenetic analysis of *Salmonella* spp. Among different isolates in different countries.
qualitative comparison of the conventional PCR assay with bacteriological culture showed a greater efficiency of PCR in terms of sample processing time and the time needed for obtaining results (36 to 48 hours), compared to the culture technique (5-6 days).

The sequencing of the product obtained confirms that the PCR method detects the invA gene of different serovar of Salmonella enterica, which guarantees the specificity of the PCR assay and is also a requirement for the validation for the diagnosis of pathogens in different clinical isolates.

InvA is a gene that is shared by all Salmonella species; as a result, it is frequently employed as a genetic target that is selective for the detection of Salmonella strains (González-Escalona et al., 2012). Furthermore, it has been reported that invA has mutation rates comparable to housekeeping gene indicating that it is a potential candidate pertaining to tests for detection using PCR (Boyd et al., 1997). invA sequences that can be obtained from the NCBI (23 global unique sequences, with 2 additional local S. enterica subsp. enterica typhimurium and S. enterica subsp. Enterica enteritidis that were sequenced throughout the course of this research, due to the fact that sequences for these serovar were not readily accessible to the public. A phylogenetic tree was generated using the maximum likelihood method with the core genome sequences of 23 reference strains; nucleotides clustering, sequence type, serotype, country, and source are indicated at phylogenetic tree. Salmonella spp. isolates belonged to the following species and serovar and accession number: S. enterica serovar Enteritidis (LC730849) and S. enterica subspecies typhimurium (LC730846).

CONCLUSION

In conclusion, the PCR technique is a basic tool for the detection of Salmonella enterica, and its virulence factors, such as the invA gene, provides information ranging from the identity of a given bacterium to its virulence potential, and Sequencing of invA gene of Salmonella showed identity 99%-100% with the world level. Also, it is suggested that the presence of Salmonella spp. in camel needs monitoring in order to minimize the risks of infection exposed the human beings.

DECLARATIONS

Corresponding author
Correspondence and requests for materials should be addressed to Dr. Afaf Abdulrahman Yousif; Email: afaf.a@covm.uobaghdad.edu.iq

Data availability
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors’ contribution
All authors contributed equally in all details of this paper.

Acknowledgements
This work was supported by Dept. of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad, Iraq.

Consent to publish
All authors agree to publish the article “Isolation and molecular identification of invA gene of Salmonella spp. in dromedary camels” in an online journal of animal and feed research.

Competing Interests
The authors declare no competing interest.

REFERENCES


