Phylogenetic analysis of Salmonella *species isolated from cows, buffaloes, and humans based on* gyrB *gene sequences*

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REGULAR ARTICLES

Phylogenetic analysis of *Salmonella* species isolated from cows, buffaloes, and humans based on *gyrB* gene sequences

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Abstract

This study aimed to investigate the role of dairy cows and buffaloes as reservoirs of nontyphoidal salmonelloses (NTS), to reveal the occurrence of NTS among dairy workers and children with acute diarrhea and to study the *gyrB* gene phylogenetic relations of the obtained *Salmonella* strains, 300 samples were chosen randomly from clinically infected animals, including 100 feces and 50 raw milk from buffaloes and cows. Five hundred samples were chosen randomly from healthy animals, including 150 feces and 100 raw milk from buffaloes and cows. A total of 160 stool samples were randomly chosen from healthy workers (60) and children with acute diarrhea (100). S*almonella* species were isolated from the examined samples and identified by polymerase chain reaction. Sequencing and phylogenetic analyses of *gyrB* gene were also performed. *S. enteritidis* and *S.*typhimurium were isolated from 0.5% (2/400) of the cows and buffaloes, respectively. Dairy workers were found to be at greater risk of exposure to *Salmonella* infection (5%) than children (1%). *S. enteritidis* was isolated from 1.7% (1/60) of dairy workers. *S.* typhimurium was isolated from 3.33% (2/60) and 1% (1/100) of dairy workers and children, respectively. Phylogenetic analysis of *Salmonella* species *gyrB* gene sequences from both animals and humans falls inside one clade, and all of them were closely related to each other with less significant genetic distance (99.9:100). In conclusion, cows and buffaloes act as reservoirs of *Salmonella* infection in dairy farms in Egypt and contribute a risk of zoonotic transmission to human.

Keywords *Salmonella* · *gyrB* gene · Zoonosis · Cows · Buffaloes

Introduction

Nontyphoidal salmonelloses (NTS) are considered one of the global foodborne zoonoses responsible for 93 million cases of gastroenteritis annually (Majowicz et al. 2010).The genus *Salmonella* involves three species, *S. enterica*, *S. bongeri*, and *S. subterranean* (Su and Chiu 2007). Based on serologic typing of the somatic (O) and flagellar (H) antigens, *S. enterica* is classified into more than 2500 serovars (Su and Chiu 2007). *S. enterica* is responsible for the majority of *Salmonella* infection in humans (Chen et al. 2013). *S. typhimurium* and *S. enteritidis* are predominately the most

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common serovars isolated from both animals and man worldwide (Tennant et al. 2016).

Livestock animals are recognized as reservoirs for NTS (Wales et al. 2010). Salmonellosis in animals is usually asymptomatic; however, animals develop disease when exposed to stress (Radke et al. 2002). Zoonotic transmission occurs through direct or indirect contact with animals, consumption of milk, meat, and/or contaminated water (Álvarez-Fernández et al. 2012). In well-developed countries, NTS gastroenteritis in human is usually self-limiting (Gordon 2008); however, in developing countries, NTS are responsible for invasive salmonellosis in human with a case fatality rate of 20–25% (Gordon 2008; Reddy et al. 2010). It has been estimated that 3.4 million cases of invasive NTS occur each year (Ao et al. 2015). Invasive salmonellosis is considered a public health problem, especially with the emergence of beta lactam-resistant NTS serovars (Chiou et al. 2014).

Egypt depends mainly on the raising of cattle and buffaloes for milk and meat production. Cattle and buffaloes make up about 23%of total agricultural value, a total of 73.5 billion EGP of which 66% is meat production and 34% milk production (FAO 2017). NTS organisms are the most frequent bacterial etiologic agents isolated from animals and humans in Egypt; however, the true prevalence is underestimated (Zahran and El-Behiry 2014; Maysa and Abd-ELall 2015).Therfore, the aim of this study was based on three objects: the first is to investigate the role of dairy cows and buffaloes as reservoirs of NTS; the second is to reveal the occurrence of NTS among dairy workers and children with acute diarrhea; and the third is to study the *gyrB* gene phylogenetic relations of the obtained *Salmonella* strains.

Materials and methods

Study area

This study was carried out in the period ranging from April 2014 to December 2016 in Asyut Governorate (situated 361 km south Cairo, Egypt, and located at 27°11′00″N 31°10′00″E).

Sample collection

A total of 800 animal samples were chosen randomly from dairy farms in Asyut Governorate, Egypt. Three hundred samples were chosen from clinically infected animals, including 150 buffaloes (100 feces and 50 raw milk) and 150 cows (100 feces and 50 raw milk). Five hundred samples were chosen from healthy animals, including 250 buffaloes (150 feces and 100 raw milk) and 250 cows (150 feces and 100 raw milk). Fecal swabs were collected directly from the rectum of buffaloes and cows. Milk samples were collected directly from the udder of animals in clean, dry, and sterile sampling container.

A total of 60 stool samples were randomly chosen from healthy workers in the investigated dairy farms who agreed to submit stool samples for examination. Moreover, a sum of 100 random stool samples was chosen from children with acute diarrhea from gastroenterology unit of Assiut Children University Hospital. Informed consent was obtained from children's families before including children in this study.

Samples were obtained under complete aseptic conditions and transferred in ice box without delay to the laboratory for bacteriological examination. The study was conducted in the Department of Animal Hygiene and Zoonoses, Faculty of Veterinary Medicine, Assiut University, Asyut, Egypt.

Isolation of *Salmonella*

Fecal swab or 1 ml of milk sample was enriched in 9 ml of buffered peptone water and incubated for 24 h at 37 °C. A portion of the pre-enriched culture (0.1 ml) was enriched in 10 ml of Rappaport and Vassiliadis broth (Biolife) and incubated at 42 °C for 24 h. One loopful of each enriched broth was aseptically streaked onto Xylose lysine deoxycholate agar (Biolife) and incubated at 37 ± 1 °C for 24–48 h. Black colonies with red background were selected and maintained on nutrient agar (World Health Organization 2010). Presumptive *Salmonella* colonies were biochemically identified as *Salmonella* using API 20E strip kit (bioMerieux ® Inc., France) according to the manufacture procedure.

Serological identification

Salmonella isolates were serotyped by slide agglutination technique using polyvalent and monovalent antisera according to Kauffman–White scheme for the determination of somatic (O) and flagellar (H) antigens by using *Salmonella* antisera (DENKA SEIKEN Co., Japan) (Kauffmann and Edwards 1952).

Preparation of chromosomal DNA

Salmonella chromosomal DNA was extracted using PrepMan Ultra Sample Preparation Reagent Quick Reference kit (Applied Biosystems P/N 4318924 Rev B) according to the manufacturer's instructions. Briefly, bacterial colony was cultured onto nutrient agar plate and incubated overnight at 37 °C for 24 h. A small loopful from a separate colony was selected from the culture plate and suspended in 100 μl of the PrepMan Ultra Sample Preparation Reagent in a microcentrifuge tube, mixed well by vortex for 10 to 30 s. The microcentrifuge tube was placed in a heat block (95 °C) for 10 min, then allowed to cool to room temperature for 2 min, and spinned at the highest speed for 3 min. A 50 μl of the supernatant were transferred from the spin tubes into a new set of labeled microcentrifuge screw cap tubes, and the remaining supernatant was discarded.

Amplification of gyrB gene

Polymerase chain reaction (PCR) was performed using PCR Master Mix Kit (Qiagen) in a total reaction volume of 25 μl with 12.5 μl of master mix, 5 μl of template DNA, 5.5 μl of grade water, and 1 μl of each primer UP1 and UP2. PCR condition was performed as described before (Fukushima et al. 2002) as following, an initial denaturation step of 95 °C for 5 min and 30 cycles of 96 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and then a final step of 72 °C for 7 min for the last cycle. The PCR products were analyzed by 1.5% agarose gel electrophoresis for 120 min at 80 V in TBE (Tris base–boric acid-EDTA) buffer. The electrophoresis products were visualized by UV trans-illuminator (Biometra) and photographed by Gel Documentation System including BioDocAnalyze (BDA) software for measuring and analyzing the PCR results.

Sequencing of gyrB gene

DNA sequencing was performed by the dideoxy chain termination method using a BigDye® Terminator v3.1Cycle-sequencing kit according to the manufacturer's instructions. PCR product was sequenced using the primers UP1S and UP2Sr (Fukushima et al. 2002). The reaction mixture included 4 μl BigDye® Terminator, 4 μl 5X sequencing buffer, 5 μl PCR product, 1 μl of each primer, and up to 20 μl nuclease-free water. Mixture was amplified as follows: initial denaturation step of 95 °C for 5 min, 40 amplification cycles including denaturation 96 °C for1 min, annealing 60 °C for1 min, and extension 72 °C for 1 min, and final extension was performed at 72 °C for 7 min using BigDye® XTerminator™ Purification Kit: 45 μl of SAM TM solution was added to each well.

BigDye® XTerminator™ solution (10 μl) was added to each well using wide bore pipette tips. The plate was sealed with heat seal film, and the contents of the plate were thoroughly mixed for 30 min. The plate was centrifuged at 1000 × g for 2 min, and the plate was run on 3500 Genetic Analyzer–Applied Biosystems DNA Sequencer using BigDye® XTerminator™ run module. Sequence reactions were analyzed on a PRISM 310 genetic analyzer (Applied Biosystems). The obtained sequences were compared with other *Salmonella* sequences registered in the gene bank, and the statistical significance of matches was calculated using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic analysis

The nucleotide divergence percent and the similarity of the nucleotide sequences were determined by using the DNASTAR's MegAlign sequence alignment software. Multiple alignments of nucleotide sequences obtained in this study together with other reference *Salmonella* sequences registered in the gene bank were performed using the Clustal X program, and phylogenetic tree was constructed by neighbor joining method. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. This analysis involved 21 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1262 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). *E. coli* (AB009855) served as an out group.

Statistical analysis

Data was analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). *P* values < 0.05 were considered significant.

Results

S. enteritidis was isolated from 0.5% (2/400) cows represented by two isolates in fecal matter and milk from the same animal with percentages of 0.4% and 0.7%, respectively (Table 1). *S. typhimurium* was recovered from 0.5% (2/400) buffaloes, and it was detected in fecal matter only with a percentage of 0.8% (Table 1). *S. enteritidis* was isolated from 0.8% (2/250) healthy cows, and we could not isolate it from diarrheic cows, and the result was not statistically significant (Table 1). On the contrary, 1.3% (2/150) diarrheic buffaloes were positive for *S. typhimurium*, and none of the healthy buffaloes carried *Salmonella*, and the result was not statistically significant (Table 1). There was no difference in exposure ($p > 0.05$) to *Salmonella* infection between dairy workers (5%, 3/60) and children (1%, 1/100) (Table 1). *S. enteritidis* was isolated from 1.7% (1/60) dairy workers. *S. typhimurium* was isolated from 3.33% (2/60) and 1% (1/100) dairy workers and children, respectively (Table 1).

The nucleotide sequences of *gyrB* gene of *Salmonella* isolates reported in this study were submitted to gene bank and are available under the following accession numbers: (KY427022, KY427023, KY427024, KY427025, KY427026, KY427027, KY427028, and KY427029).

S. enteritidis KY427022 (human stool) and *S. typhimurium* KY427023 and KY427025 (buffaloes fecal matter) which were isolated from the same farm were closely related (99.9:100). *S. enteritidis* (KY427028, KY427029) were isolated from milk and fecal matter of the same animal, respectively. In addition, *S. typhimurium* KY427024 was isolated from human stool from the same farm. All of these three sequences were closely related to each other. Finally, two *S. typhimurium* isolates recovered from human stool

Table 1 Occurrence of *S. enteritidis* and *S. typhimurium* in cows, buffaloes, and man

Source of samples	No. of samples	*S. enteritidis*		*S. typhimurium*		*P* value	Total
Cows		No.	%	No.	%		
Healthy	250	*2	0.8	0.0	0.0	0.5301	
Diarrheic	150	0.0	0.0	0.0	0.0		
Total	400	2	0.5	0.0	0.0		
Buffaloes							
Healthy	250	0.0	0.0	0.0	0.0	0.1400	
Diarrheic	150	0.0	0.0	2	1.3		
Total	400	0.0	0.0	2	0.5		
Fecal matter							
Cows	250	1	0.4	0.0	0.0		
Buffaloes	250	0.0	0.0	2	0.8		
Milk							
Cows	150	1	0.7	0.0	0.0		
Buffaloes	150	0.0	0.0	0.0	0.0		
Dairy workers	60	1	1.7	2	3.33	0.1487	3(5%)
Children	100	0.0	0.0	1	1		1(1%)

*source: milk, fecal matter from the same animal

(KY427026 and KY427027) from another farm and children university hospital, respectively, were also proven to be closely related (99.9) to the abovementioned sequences .

The similarity between the Egyptian isolates and other sequences obtained from the gene bank was slightly different, and the similarity ranged from 97.4 up to 99.9. Lower degree of similarity (97.7:97.8) was noted with *S. enterica* Iranian and Chinese strains (EF643611, EU146972, and DQ344541), respectively, while the similarity with Austrian strain (DQ386877) and Korean strain (EU014643) was 98.3:98.4. On the other hand, the highest degree of similarity was identified with other Chinese strains of *S. enterica* (EF064844 and EF064845) (99.3:99.5). All *Salmonella* sequences were completely separated from the clade of *E. coli* which was used as an out rooted group with significant genetic differences 46.8:47.2 (Fig. 1).

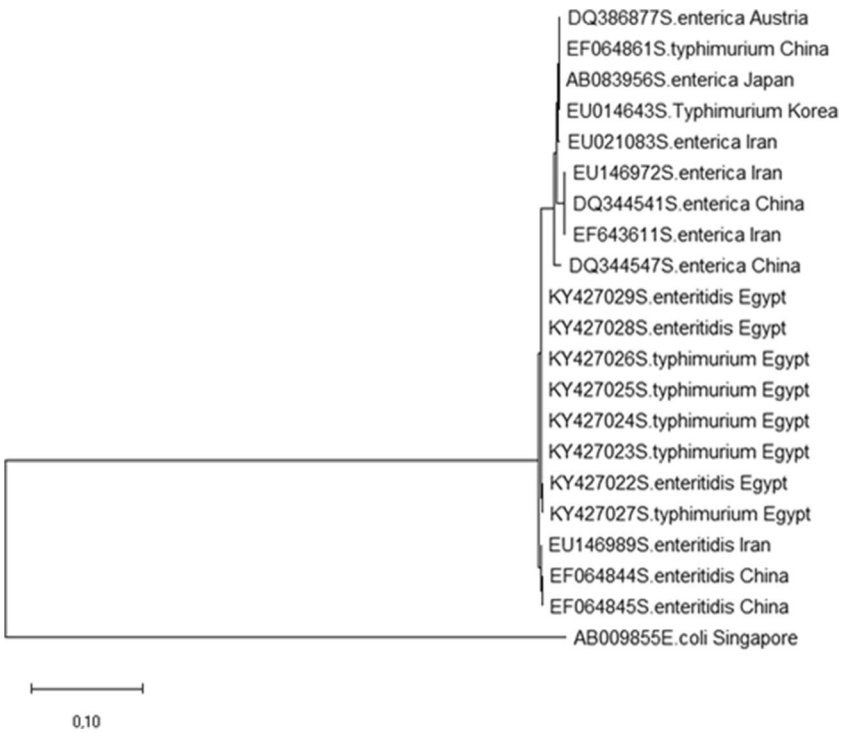

Fig. 1 Phylogenetic analysis of *gyrBgene* of the isolated *Salmonella* species and the reference *Salmonella* species

Discussion

Salmonellosis continues to be one of the major economic diseases in dairy farms associated with productivity loss in animal industry (Malkawi and Gharaibeh 2004). In the present study, *Salmonella* species were isolated at the same rate from cows and buffaloes (Table 1). On the contrary, higher infection rate was reported in buffaloes (5.7%) than in cows (5%) in another study in Egypt (Zahran and El-Behiry 2014). Much higher percentage (22.5%) of *Salmonella* isolation was reported in cows in another study in the USA (Cummings et al. 2009). Concerning the infection in buffaloes, much higher percentage (2.16%) was reported in another study in Egypt (Hassanain 2008). *S. enteritidis* was the prevalent species among cows; meanwhile, *S. typhimurium* was the prevalent species among buffaloes. Conversely, other investigators reported that nearly similar serovars of *Salmonella* were recovered from both cows and buffaloes (Aydin et al. 2001). The variation in the reported *Salmonella* species in cows and buffaloes in different studies may be attributed to the difference in the prevalent species in the studied area.

Concerning the recovery of *S. enteritidis* from healthy cows in this study likewise, it is well-documented that animals may act as carriers of *Salmonella* species and the herd carrier status is positively correlated to the herd size (Hume et al. 2004). The inability to isolate *Salmonella* from cows with diarrhea may be attributed to infection of cows by another enteric microorganism rather than *Salmonella* or because of the intermittent shedding of *Salmonella* in the feces of animals. Contrary to this finding higher *Salmonella* infection rate was recovered from diarrheic cows (7.69%) compared to apparently healthy cows (0.97%) (Mohamed et al. 2011). The differences in the isolation rates reported in different studies may be attributed to differences in the ecological conditions, type of samples, and/or culture methods (Enticott 2003).

Interestingly *S. enteritidis* was isolated from the feces and milk of the same animal (Table 1). As we rule out the possibility of contamination from the surrounding environment as we collect all the samples under complete aseptic condition from the udder, so, it is properly concluded that *S. enteritidis* was colonizing the udder. Likewise, it has been documented that *Salmonella* species can colonize the udder and shed in milk (Acha and Szyfers 2001).

Buffaloes are infected with *Salmonella*, and the infection varies from subclinical to clinical disease (Hassanain et al. 2010). In this study, infection of diarrheic buffaloes result is compatible with the results showed in another study (Davies et al. 2004). On the other hand, much higher percentage (9.2%) was noted elsewhere (Zahran and El-Behiry 2014).

In comparison to the infection of dairy workers in this study, higher prevalence rate (7%) of *Salmonella* was reported in healthy dairy handlers, respectively, in other studies (Maysa and Abd-ELall 2015). Concerning infection in children (Table 1), higher prevalence rate (6.2%) was previously recorded in infant and children with acute diarrhea, respectively (Beyene and Tasew 2014).

Phylogenetic analysis of *Salmonella* species *gyrB* gene sequences of 4 strains of animal origin and 4 strains of human origin in this study falls inside one clade evidencing that it belongs to the genus *Salmonella* and all of them were closely related to each other with less significant genetic distance (99.9:100) and less significant branches. Similar results were obtained in another study reporting that different species of *Salmonella* were located in one cluster by using phylogenetic analysis of *gyrB* gene. However, phylogenetic analysis of *Salmonella* species using 16SrRNA does not always share the same cluster (Fukushima et al. 2002). The similarity between the Egyptian isolates and other sequences obtained from the gene bank was slightly different, and this may be attributed to the differences of the host, source, and locality. Depending on the obtained results, phylogenetic analysis, and testing of the genetic distances, the Egyptian *Salmonella* isolates from animals and human were closely related.

Conclusion

Cows and buffaloes act as reservoirs of *Salmonella* infection in dairy farms in Egypt and contribute a risk of zoonotic transmission to human. Phylogenetic analysis revealed the circulation of closely related *Salmonella* species in animals and man. Therefore, it is necessary to apply intervention strategies to control *Salmonella* in dairy farms. Additionally, safety measures should be applied in the production of milk and milk products. Finally, increasing the awareness of dairy workers and the public is critical to prevent the zoonotic transmission of *Salmonella*.

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Authors' contributions Amal Sayed and H. Abd ElKader planned and coordinated the study. Lamiaa Ahmed carried out the practical work and shared in manuscript writing. Nagla Hassan Abu Faddan supervised the human clinical cases and shared in data analysis. Amira Al Hosary carried out the phylogenetic analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interests.

Ethics approval and consent to participate Ethical standards of the Helsinki Declaration were followed with human work in this study. The protocol and procedures employed were ethically reviewed and approved by the Scientific Research Committee and Ethics Board of Assiut University, Faculty of Medicine, Asyut, Egypt (IRB no:17300232). The study also conforms to recognized standards of Animal research applied by Assiut University.

Abbreviations *Bp*, base pair; *°C*, Celsius; *CI*, confidence interval; *E*, east; *h*, hours; *Km*, *kilometer*; *ml*, milliliter; *min*, minutes; *N*, north; *NTS*, nontyphoidal *Salmonella*; *P*, probability; *PCR*, polymerase chain reaction; µl, microliter

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