A Random Survey of Microbial Load in Dairy Products on Sale in Addis Ababa Supermarkets

Solomon H H. Mariam (solomon.habtemariam@aau.edu.et)
Addis Ababa University Addis Ababa, Shoa ETHIOPIA

Research article

Keywords: Dairy isolate, enterobacteriaceae, pasteurization, antibiotic resistance, sequence similarity

Posted Date: May 26th, 2019

DOI: https://doi.org/10.21203/rs.2.9848/v1

Abstract

Background: Food-borne infectious diseases cause huge economic loses, lowered quality of life and, in extreme cases, losses of lives. In general, these are global problems but the magnitudes differ. Pathogens involved include those of the genera Salmonellae, Escherichia, Campylobacter, Yersinia, Listeria, and Staphylococci. Infections follow consumption of contaminated foods or animal to human and human to human transmissions. Contaminated dairy products are among the top sources of food-borne infections. Most such infections come from unpasteurized dairy products, but pasteurized products are sometimes implicated. These occur because of either faulty pasteurization or due to post-pasteurization contaminations. Many factors hamper control of such infectious pathogens, including lack of vaccines, the presence of asymptomatic healthy carriers, existence of broad host range pathogens (with the hosts serving as reservoirs), resistance of pathogens to ordinary disinfectants, the long contamination-prone processes from production to consumption and increased frequency of resistance of pathogens to antibiotics. The antibiotic resistance property is hazardous in itself (whether with or without the contaminants being pathogenic) considering the potential for interspecies transfer of these resistance traits (be they chromosomal or plasmid-borne) to commensal or pathogenic bacteria in clinical settings and/or in the food chain. In low income countries, the problems are further compounded by the limited capacity for accurate diagnosis, overlap of symptoms, and the resulting (hit-or-miss) empirical treatment. Here, samples of dairy products were purchased from Addis Ababa supermarkets. The samples were subjected to enrichment and selective cultures to test the presence of and isolate gram-negative bacteria. The isolates were characterized by biochemical and molecular methods. Results: All milk samples harboured gram-negative bacilli, which are likely to constitute hazard to public health. Moreover, all of the isolates exhibited intermediate-level or higher resistance to two or more clinically relevant antibiotics. Sequence alignment showed the isolates to be most closely related to Salmonella enterica. Conclusion: This work found high level of contamination in all sampled milk. The reason(s) for this burden of contamination (either ineffective pasteurization or post-pasteurization contamination) need to be elucidated for meaningful targeted control.

Background

Members of the family *e*nterobacteriaceae are major causes of food-borne infections worldwide. The *e*nterobacteriaceae are gram-negative, non-spore-forming, facultatively anaerobic, catalase-and nitrate-positive, oxidase-negative and generally capable of growth on medum containing simple nutritional substrates as they can ferment a wide range of carbohydrates. The family includes many genera (*Escherichia*, *Shigella*, *Salmonella*, *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus*, and others). While many are part of the normal flora of humans and animals, others are always associated with disease (e.g., *Salmonella Typhi*, *Shigella* species, *Yersinia pestis*) and still others can be occasionally pathogenic – either opportunistically or following acquisition of virulence factors [1]. The *e*nterobacteriaceae possess complex antigenic structures and produce a variety of toxins and other virulence factors. Humans acquire these bacteria after consumption of contaminated foods, from contact with animals, or directly via

human-to-human transmission (especially the human-host-specific pathogens such as *S*. typhi) [2]. Foods of dairy origin account for a large share of the burden of food-borne illnesses [3, 4]

A review by Reddy et al [5] indicated that about 30% of non-malarial blood stream infections are caused by *Salmonella* species, and most of these salmonellosis infections are due to non-typhoidal *Salmonella* (NTS). The NTS, such as *S*. Typhimurium and *S*. Enteritidis, may be characterized by genomic evolution within the host or during host-to-host transmission, driven primarily by prevailing conditions (e.g., changes in host immunity, presence of antimicrobials) [6]. The epidemiology of food-borne infections is changing, including its several manifestations: emergence of new and more virulent pathogenic bacterial clades, regional or global spread, and dissemination of antibiotic resistance [7, 8]. In sub-Saharan Africa, the NTS are responsible for the vast majority of invasive *Salmonella* infections and cause bloodstream infections in both adults and children (primarily in the immune-compromised and malnourished) presenting with fever and are associated with case fatality rates of 20–25% [9, 10]. The latter study also showed that NTS infections in Ethiopia account for up to 10% of blood stream infections.

Elsewhere, reports indicate infections and disease sequel due to enteric bacterial pathogens from pasteurized dairy products [3, 11]. Such studies are rare or nonexistent in Ethiopia. Technically, it is challenging to conduct such studies, and a specially daunting task is tracing the cause-effect connections of foods implicated in such infections, which require conducting controlled investigations. These scenarios make prevention all the more feasible and realistic approach to manage the problems, but some data are needed to base actions on. This small-scale study was undertaken to provide some information on the possible health risks that could result from consumption of local pasteurized dairy products by the general public, so that the study can serve as indicator of the situation on the wider scale.

Methods

The study site Addis Ababa is the capital of Ethiopia. The city's milk supplies come from dairy cooperatives and private dairy industries, and milk pooling is the rule. The same brands of dairy products are distributed for sale at all supermarkets in Addis Ababa. The population size is estimated at 3-4 million. Moreover, it is quite an international place being the seat of many continental and global organizations and hosting more than 125 country embassies.

Samples and Culture media

Samples of dairy products (cheese, milk) were purchased from food supermarkets. These products are produced by 8 different private dairy plants. All samples analyzed in this study are declared to have been pasteurized by the producers. All food samples were subjected to pre-enrichment cultures within 1 hr of purchase. All liquid and agar media and reagents are from Becton Dickinson, unless stated otherwise.

Culture and isolation

Samples were inoculated into buffered peptone water (BPW) (Himedia, India) (10 mL sample in 10 mL BPW) and incubated for 24 hrs at 37^{o}C. Similarly 10 g of cheese was inoculated into 10 mL of BPW and incubated for 24 hrs at 37^{o}C. Then, 1 mL of each was inoculated into 9 mL selenite broth and incubated for 24 hrs at 37^{o}C. Following this, successive 10-fold dilutions were made for each sample and 30 L was inoculated onto *Salmonella-Shigella* (SS) agar and incubated for 24 hrs at 37^{o}C. Black-coloured single colonies were picked and inoculated into Luria-Bertani (LB) broth and grown aerobically at 37^{o}C for 24 hrs. These procedures (culture on SS agar and growth of selected colonies on SS agar) were repeated. Samples were also inoculated onto MacKonkey agar. Finally, broth cultures were pelleted and the pellets stored in LB broth containing 15% glycerol in multiple aliquots to serve as stocks for subsequent studies.

Identification

A combination of cultural, biochemical and molecular tests was used for identification. Each sample was grown on LB agar and gram-stained to test whether each sample contained garm-positive or -negative organisms. Each cultural and biochemical test was conducted two independent times

Catalase test – colonies grown on LB agar for 24 hrs were separately placed on slides and 1-2 drops of 30% H_2O_2 added.

Motility test - Screw-cap tubes (13 by 100 mm) containing 5 mL of sterilized motility test medium were inoculated in the center to a depth of 0.5 inches with a colony grown for 24 hrs. The tubes were then incubated at 35^{o}C or at room temperature for 24 hrs.

Urease test – Urea broth was prepared and filter-sterilized through 0.45m pore size filters. Three mL volumes were then dispensed into 13 by 100 mm sterile screw-cap tubes. Each isolate was inoculated into the broth using a sterile loop and incubated in water bath set to 35^{o}C for 24 hrs, with observations made at 1 hr and 2 hrs.

Citrate utilization test – Simon's citrate agar (OXOID) was freshly-prepared in glass tubes, sterilized, and placed at an angle to form a butt and slant. Each tube of medium was inoculated with 20 hrs grown colony of the dairy isolates and the tubes were loosely-capped and incubated at 35^{o}C for 24 hrs or longer, with daily observation for changes in colour of the media.

Kligler iron agar (KIA) test – KIA agar was prepared and moltened by heating in water bath. Five mL was dispensed into sterile screw-cap tubes and autoclaved at 121^{o}C for 15 minutes. The tubes were then placed in a slanted position to form a butt and slant during cooling. A 24 hr-grown colony of each isolate was stabbed deep into the center of the butt and the surface of the slant was also gently streaked. The inoculated tubes were loosely-capped and incubated at 35^{o}C for 24 hrs.

DNA extraction and Sequencing of 16S rRNA - DNA was extracted from the isolates for use in polymerase chain reaction (PCR) and sequencing. DNA concentrations were in the range of 540-1200 g/L. Sequencing of the 16S rRNA gene was performed for testing sequence similarities to 16S rRNA gene

sequences obtained from the National Center for Biotechnology Information (NCBI) database. Each sequence was pair-wise blasted with different 16S rRNA gene partial sequences, and multiple sequence alignments (MSA) were also performed and a basic phylogenetic tree generated with the MSA tool T-Coffee using 16S rRNA gene partial sequences of the study isolates and type strains. The 16S rRNA gene partial sequences of strains (subspecies of *S*. enterica and *Lactococcus lactis*, *E. coli*, *S. aureus*, etc) were used for sequence alignment.

Antibiotic sensitivity tests (ASTs) - ASTs to available antibiotics were conducted by disk diffusion, and guidelines of the Clinical Laboratory Standards Institute (CLSI) [12] were used. Briefly, broth cultures of the isolates were grown until culture turbidities matched McFarland 0.5 standard. Sterilized Mueller-Hinton agar was poured into 100 mm plates (20 mL/plate) and when firmly solidified were streaked over the entire agar surface with sterile cotton swab that had been dipped into the bacterial broth culture. Antibiotic disks (Abtek, England), 4 per plate, were dispensed onto the agar surface 15 minutes after the streaking. The plates were incubated at 35^{o}C for 24 hrs. The following antibiotic disks were used: chloramphenicol (Chl, 30g), tetracycline (Tet, 30g), gentamycin (Gent, 10g), ciprofloxacin (Cipro, 5g), nitrofurantoin (Nitr, 300g), ampicillin (Amp, 10g), erythromycin (Ery, 15g), and ceftriaxone (Ceftr, 5g). Inhibition zone diameters were measured in millimeters.

Some procedures (e.g., testing of the isolates sensitivity or resistance to antibiotics, alignment of sequences) were performed without regard to the presumed identities of the isolates.

Quality control - *Escherichia coli* (American type culture colletion [ATCC]) 25922, *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis* ATCC 29906, *Klebsiella pneumonia* ATCC700603, Salmonella Enteritidis ATCC 13076 and un-inoculated medium were used as positive or negative controls as appropriate in the different tests.

Results

The isolates were all gram-negative rods. Black colonies on SS were selected, amplified in broth and stored frozen in aliquots. All isolates were obtained from pasteurized milk. While milk samples from all producers gave gram-negative growth, some cheese samples were also cultured but did not show growth.

On the motility test medium, isolates, 1, 3, 5, 6, 9, 12, and 15 were motile, while isolates 4, 7 and 24 did not exhibit motility. All of the isolates were found to be catalase-positive and all isolates were negative for the urease test. Cultural and biochemical test results are shown in Table 1.

The results of sequence alignment showed that each isolate was most closely related to the *S*. enterica subspecies, with similarities ranging from 91% to 99% (Table 2). Overall, isolate 1 showed the highest nucleotide similarity to the different *S*. enterica subspecies (98-99%). Conversely, the relations with most of the non-salmonellae sequences were found to be much lower (77-85%) (Table 2). All of the isolates were related more distantly to the *Enterococci* and *Lactococci*, with the percent similarities being 77 and 80%. Some isolates (1, 5 and 20) showed no significant similarity to the two enterococci and the two

lactoococci (Table 2). On the other hand, the dairy isolates had no more (or less) nucleotide similarity to each other than they had to the *S.* enterica species, with percent similarities ranging between 91 and 98 (data not shown). A basic phylogenetic tree is shown in Figure 1. There are several other gram-negative isolates found in this study, but were not further analyzed.

[Table 2]

[Figure 1] Figure 1. Phylogenetic tree generated with multiple sequence alignment using 16S rRNA gene

partial sequences of the study isolates and type strains. (“Sample” is synonymous to “Isolate” in

the text; e.g., Sample 1 is the same as Isolate 1).

Almost all isolates were found to be susceptible to both Tet and Chl. However, isolate 3 was resistant to four antibiotics: Tet, Chl, Cipro and Amp. All of the rest of the isolates were intermediate-resistant to Cipro, and all isolates were resistant to Amp (Table 2). All isolates were resistant to Ery, but this is due to intrinsic resistance. Many of the isolates are multiple drug-resistant. Isolate 3 appears to be specially notorious – being resistant to most of the antibiotics tested. It was not possible to determine the susceptibility/resistance of the isolates to Ceftr, since the CLSI guideline gives categories for 30 g disk, but 5 g disks were used here; however, the measured zone diameters are shown in Table 3.

Discussion

There are numerous reports, including from industrialized countries, implicating dairy products as causing infections in humans and resulting in illnesses, hospitalizations and even deaths. These include both pasteurized and unpasteurized dairy products that harbour pathogens. The situation in sub-Saharan Africa appears to be much worse, but it remains to be proven whether, due to frequent and higher levels of exposure, people are already “naturally vaccinated” because otherwise one would think even more losses of lives must be occurring. There is also resistance to recommendations to avoid unpasteurized dairy products and misconception about the health benefits of raw milk [13, 14]. Meanwhile, the infections (and the probable ensuing diseases) continue to spread, which can be even more disabling when antibiotic resistance is added to the equation.

One likely explanation for the finding of pathogens in pasteurized dairy products is defective pasteurization. An alternative explanation is contamination of the products during post-pasteurization handling [15, 16]. A separate and independent reason could be that the pathogens survived the pasteurization; however, that should be a rarer reason if at all one. At this point in this study, it is difficult to say whether the pasteurization processes were less than optimal, or which of these possibilities could be an explanation for the present findings. It is possible that at least some of these isolates pose risk to consumers’ health.

The reason why some of the isolates are non-motile is not known. However, motility-defective (aflagellate or flagellar mutants) strains are known to exist, with reduced ability in vivo to invade deeper tissue or

invade other organs such as the liver and spleen [17, 18]. While almost all cultural and biochemical tests turned out results compatible with those of salmonellae, the KIA test results were difficult to interpret for most isolates – not fitting entirely into known descriptions of KIA metabolism tests. The results of the two independent KIA tests were identical for each isolate. In fact, the results of the KIA tests alone are inconclusive to speculate on the identities of the isolates. They could be metabolic variants; but, that remains to be verified.

The 16S rRNA gene, which is about 1500 bp long, is known to be conserved, with variable sequences interspersed in between. These features make it ideal target for amplification and sequence comparisons for species-level identification of clinically important bacteria [19, 20]. However, there is no consensus on the use of this sequence in phylogenetic analyses. On the other hand, it has been suggested that the first 500 bp sequences of the 16S rRNA gene can give similar species-level discriminatory power as the entire 1500 bp sequence in more than 90% of comparisons in a given sequence; and the entire 1500 bp sequence can give similar phylogeny as the whole genome [21, 22]. For comparisons involving the 16S rRNA gene, sequence similarity scores of 99% and 97% were suggested to give species- and genus-level distinctions, respectively [23, 24]. Based on this, it may be said that isolates such as 1, 3, 5, 7, 15 and 24 belong to the *Salmonella* genus. On the basis of the 16S rRNA gene sequences, it was not possible to assign a species group to the bacterial isolates in this study, except perhaps for isolates 1, 5 and 7. Indeed, the 16S rRNA gene sequence has low power of resolving to the species level [24]. These isolates may be new species, undescribed in the database. However, the isolates may be ancestrally related to *S.* enterica serovar Enteritidis and *S.* enterica serovar Typhimurium.

This study has some limitations. It will be necessary to investigate the full virulence potentials of the isolates, which was not addressed in this work. The small number of antibiotics tested here is also a limitation. It is possible that some other gram-negative bacterial species escaped detection in this work. Definitive identification of the isolates is needed; serotyping would have helped.

Conclusions

Though it was not possible to identify the isolates in this study to the species level, there should be little doubt that these and similar isolates constitute hazard to the health of consumers. The antibiotic resistance phenotype alone is enough to pose such hazard – whether or not these isolates turn out to be pathogenic by other mechanisms, since there is the potential for interspecies transfer of these resistance traits to commensal or pathogenic bacterial species in vivo or in the food chain [25, 26, 27, 28, 29]. This study's findings have significance from public health point of view; and importantly, these call for inspection of both the pasteurization processes in the dairy plants and the manners of post-pasteurization handling of the products, defects in either one or both of which can explain the reasons for these findings.

Abbreviations

Amp – ampicillin, ATCC – American type culture collection, BPW - buffered peptone water, Ceftr – ceftriaxone, Chl – chloramphenicol, Cipro – ciprofloxacin, Clinical Laboratory Standards Institute, Ery – erythromycin, Gent – gentamycin, KIA - Kligler iron agar, LB - Luria-Bertani, MSA - multiple sequence alignments, Nitr – nitrofurantoin, NCBI - National Center for Biotechnology Information, NTS - Non-typhoidal *Salmonella,* PCR - polymerase chain reaction, SS - *Salmonella-Shigella*, Tet - tetracycline .

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable

Availability of data and materials The sequences of isolates of this study will be made available upon request.

Competing interests

None

Funding This work was supported by funding from Addis Ababa University. The funder had no role in the manner of conduct or outcome of the study.

Author contributions SHM designed, conducted the studies, analyzed the data and wrote the manuscript.

Acknowledgments The author thanks Biruk Yeshitla and Melaku Yidnekachew for technical and material assistance.

References

1. Brown SP, Cornforth DM, Mideo N. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends Microbiol. 2012;20:336-42.
2. Bäumler A, Fang FC. Host Specificity of Bacterial Pathogens. Cold Spring Harb Perspect Med. 2013;3(12):a010041.
3. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, Griffin PM. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008. Emerg Infect Dis. 2013;19:407-15.
4. Hoffmann S, Devleesschauwer B, Aspinall W, Cooke R, Corrigan T, Havelaar A, Angulo F, et al. Attribution of global food borne disease to specific foods: Findings from a World Health Organization structured expert elicitation. PLoS ONE. 12(9):2017; e0183641.l
5. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10(6):417–32.
6. Tanner JR, Kingsley RA. Evolution of Salmonella within Hosts. Trends Microbiol. 2018;26(12):986–98.
7. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, Harris D, et al. Epidemic multiple

drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. Genome Res. 2009;19(12):2279-87.
8. Klemm EJ, Wong VK, Dougan G. Emergence of an extensively drug-resistant Salmonella enterica serovar Typhi clone harboring a promiscuous plasmid encoding resistance to fluoroquinolones and third-generation cephalosporins. MBio. 2018;9:e00105–18.
9. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal Salmonella disease: an emerging and neglected tropical disease in Africa. Lancet. 2012;379:2489–99.
10. Uche IV, MacLennan CA, Sau A. A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal Salmonella (iNTS) Disease in Africa (1966 to 2014). PLoS Negl Trop Dis. 2017;11(1):e0005118.
11. Ryan CA, Nickels MK, Hargrett-Bean NT, Potter ME, Endo T, Mayer L, et al. Massive outbreak of antimicrobial-resistant salmonellosis traced to pasteurized milk. JAMA. 1987;258:3269–74..
12. Clinical Laboratory Standards Institute (CLSI), Performance standards for antimicrobial susceptibility testing twenty second informational supplement, Document M100-S22, Clinical Laboratory Standards Institute, Wayne, Pa, USA, 2012. 27th edition, January 2017.
13. Rosenbaum S. Law and the Public's Health. Public Health Reports. September–October, 2014; 129:455-7.
14. Amenu K, Wieland B, Szonyi B, Grace D. Milk handling practices and consumption behavior among Borana pastoralists in southern Ethiopia. J Health Popul Nutr. 2019;38:
15. Olsen SJ, Ying M, Davis MF. Multidrug-resistant Salmonella Typhimurium Infection from Milk Contaminated after Pasteurization. Emerg Infect Dis. 2004;10:932-5.
16. Langer AJ, Ayers T, Grass J, Lynch M, Angulo FJ, Mahon BE. Nonpasteurized Dairy Products, Disease Outbreaks, and State Laws—United States, 1993–2006. Emerg Infect Dis. 2012;18(3):385-91.
17. Robertson JMC, McKenzie NH, Duncan M, Allen-Vercoe E, Woodward MJ, Flint HJ, Grant G. Lack of flagella disadvantages Salmonella enterica serovar Enteritidis during the early stages of infection in the rat. J Med Microbiol. 2003;52:91–9.
18. Yim L, Betancor L, Martínez A, Bryant C, Maskell D, Chabalgoity JA. Naturally Occurring Motility-Defective Mutants of Salmonella enterica Serovar Enteritidis Isolated Preferentially from Nonhuman Rather than Human Sources. Appl Environ Microbiol. 2011;77:7740–48.
19. Fredericks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev. 1996;9(1):18–33.
20. Watts GS, Youens-Clark K, Slepian MJ, Wolk DM, Oshiro MM, Metzger GS, et al. 16S rRNA gene sequencing on a benchtop sequencer: accuracy for identification of clinically important bacteria. J Appl Microbiol. 2017;123(6):1584–96.
21. Clarridge JE. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev. 2004;17(4):840-62,
22. Hong S, Farrance CE. Is it Essential to Sequence the Entire 16S rRNA Gene for Bacterial Identification? Amer Pharmac Rev. November/December 2015;1-7.
23. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral J-P, Raoult D. 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. J Clin

Microbiol. 2000;38(10):3623–30.
24. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol. 2007;45(9):2761-4.
25. Verraes C, Boxstael SV, Meervenne EV, Coillie EV, Butaye P, Catry B, de Schaetzen M-A, et al. Antimicrobial Resistance in the Food Chain. Int J Environ Res Public Health 2013;10:2643-69.
26. Aviv G, Rahav G, Gal-Mor O. Horizontal Transfer of the Salmonella enterica Serovar Infantis Resistance and Virulence Plasmid pESI to the Gut Microbiota of Warm-Blooded Hosts. MBio. 2016;7(5):e01395-16.
27. Pornsukarom S, Thakur S. Horizontal Dissemination of Antimicrobial Resistance Determinants in Multiple Salmonella Serotypes following Isolation from the Commercial Swine Operation Environment after Manure Application. Appl Environ Microbiol. 2017;83(20):e01503-17.
28. Conwell M, Daniels V, Naughton PJ, Doole JSG. Interspecies transfer of vancomycin, erythromycin and tetracycline resistance among Enterococcus species recovered from agrarian sources. BMC Microbiol. 2017;17(1):19.
29. Lerminiaux NA, Cameron ADS. Horizontal transfer of antibiotic resistance genes in clinical environments. Can J Microbiol. 2019;65:34–44.

Tables

The tables could not be inserted due to technical limitations. They have been included as image files in the Supplementary files section below.

Figures

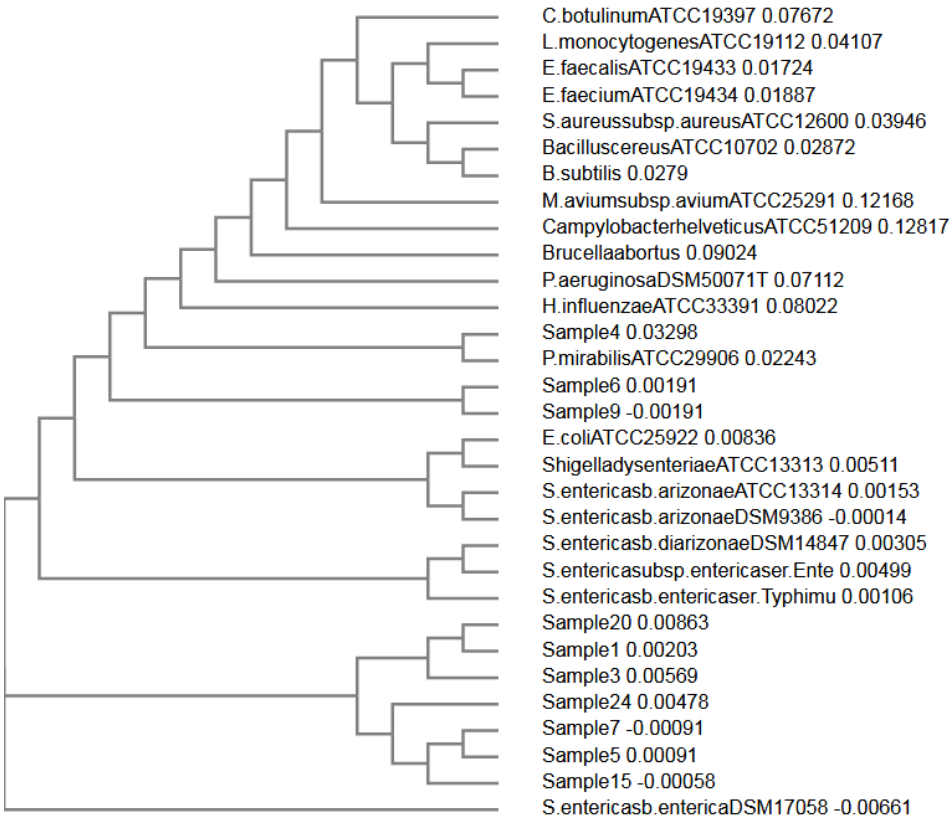

Figure 1

Phylogenetic tree generated with multiple sequence alignment using 16S rRNA gene partial sequences of the study isolates and type strains. (“Sample” is synonymous to “Isolate” in the text; e.g., Sample 1 is the same as Isolate 1).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.jpg
- supplement2.jpg
- supplement3.jpg