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Characterization of *Salmonella* isolated from apparently healthy slaughtered cattle and retail beef in Hawassa, southern Ethiopia

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ABSTRACT

The present study was conducted to determine the occurrence, serotype distribution and antimicrobial resistance of *Salmonella* serotypes in slaughtered cattle, slaughterhouse environment and retail beef. Cecal content (CC), mesenteric lymph nodes (MLN), spleen and carcass swab (CS) samples (each, n = 150) were collected from 150 cattle slaughtered at Hawassa municipality slaughterhouse. Floor swab specimens (SHFS) were collected on 11 occasions from the slaughterhouse, and 100 beef samples were collected from 100 butcher shops. The samples were cultured for *Salmonella,* following standard procedures. A total of 14 *Salmonella* isolates belonging to 3 serotypes namely *Salmonella enterica* serotype Muenchen (4 isolates), *S. enterica* serotype 1,4,5,12:i:- (5) and *S. enterica* serotype Korovi (5) were recovered. All of the 5 *S. enterica* serotype 1,4,5,12:i:- isolates belonged to phage type 120. Four (2.7%) of the slaughtered cattle carried *Salmonella* in their CC and/or MLN, while none of the spleen samples were positive for *Salmonella*. *Salmonella* was isolated from four (2.7%) CC and two (1.3%) MLN samples. Out of the total of 150 CS samples, two (1.3%) were found contaminated with *Salmonella*, while 4 (4%) of the 100 beef samples obtained from butcher shops yielded *Salmonella*. Two of the 11 (18%) SHFS were positive for *Salmonella*. All the four isolates from beef were *S. enterica* serotype Muenchen, while both of the isolates from MLN were *S. enterica* serotype1,4,5,12:i:-. Both *S. enterica* serotype1,4,5,12:i:- and *S. enterica* serotype Korovi were isolated from CC, CS and slaughterhouse environment. All the 14 isolates recovered during the study were tested and found pan-susceptible to a panel of 14 antimicrobials. The present study helped to update the information on the occurrence, serotype distribution and antibiogram of *Salmonella* in slaughter cattle and beef in Ethiopia.

1. Introduction

Salmonellosis is among the main foodborne diseases in humans with worldwide distribution. The global burden of non-typhoidal *Salmonella* gastroenteritis has been estimated to be 93.8 million cases each year with 155,000 deaths (Majowicz et al., 2010). Animals are the principal reservoirs of *Salmonella* infection to humans (Acha and Szyfres, 2001; EFSA/ECDC, 2014a) although foods of vegetable origin contaminated by animal products, human excreta or contaminated utensils have been implicated as the vehicle of human salmonellosis (Acha and Szyfres, 2001). Foods containing products from farm animals, including cattle, are important sources of human *Salmonella* infections (Smerdon et al., 2001; van Duijkeren et al., 2002). Beef has been implicated as a vehicle of human infection in several outbreaks (CDC, 1995; Davies et al., 1996; Roels et al., 1997). Contamination of carcass by *Salmonella* may occur at abattoirs from carrier animals excreting/harboring the organism (Samuel et al., 1980; Nabbut and Al-Nakhli, 1982; Morgan et al., 1987; Berends et al., 1997; Arguello et al., 2012) and by contaminated abattoir equipment and utensils (Smeltzer et al., 1980; Mannion et al., 2012). Meat available at retail outlets comes through a long chain of slaughtering and transportation, where each step may pose a risk of microbial contamination including salmonellae. The sanitary conditions of abattoir and meat shop environments play important roles in the spreading of *Salmonella* contamination (Acha and Szyfres, 2001; Mannion et al., 2012; Garedew et al., 2015).

Contamination of food with antibiotic-resistant *Salmonella* can be a major threat to public health, due partly to increased virulence and treatment failures (Helms et al., 2002; Angulo et al., 2004; Varma et al., 2005). The use of antimicrobial agents in food animals may promote development of antimicrobial resistance in *Salmonella*, with potential for transmission to humans via the food supply or other routes (Angulo et al., 2000; Threlfall, 2002).

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Even if data regarding meat-borne diseases in Ethiopia are scarce, several studies conducted in different parts of the country have shown the prevalence of *Salmonella* in cattle, cattle carcasses and beef (Nyeleti et al., 2000; Alemayehu et al., 2003; Ejeta et al., 2004; Addis et al., 2011; Sibhat et al., 2011; Alemu and Zewde, 2012). The prevalence of non-typhoidal salmonellae in humans has also been described (Aseffa et al., 1994; Mache et al., 1997; Addis et al., 2011; Eguale et al., 2015). A high level of antimicrobial resistance in *Salmonella* isolated from food animals has also been reported in Ethiopia (Alemayehu et al., 2003; Molla et al., 2003; Molla et al., 2004; Aragaw et al., 2007).

Knowledge on the occurrence and identification of sources and points of contamination are imperative for implementation of effective strategies for prevention of contamination of meat with *Salmonella*. It is also important to monitor antimicrobial resistance profile of *Salmonella* isolates for proper treatment of patients. Therefore, this study was conducted with the aim of describing the occurrence and antimicrobial resistance of *Salmonella* serotypes in slaughter cattle, slaughterhouse environment and retail beef in Hawassa, southern Ethiopia.

2. Materials and methods

2.1. Study area and animals

The study was undertaken from November 2015 to April 2016 in Hawassa town and involved the Hawassa Municipality abattoir and 100 butcher shops in the town. Hawassa is the capital city of Southern Nations, Nationalities and Peoples Regional State (SNNPRS) located about 275 km South of Addis Ababa. It is geographically positioned at 7°5′ latitude N and 38°29′ longitude E at an altitude of 1708 m a.s.l. The mean annual rainfall and temperature of the area vary from 800 to 1000 mm and 20.1–25 °C respectively. According to the Central Statistical Agency (CSA) population census report, Hawassa town had 259,803 residents in 2007 (CSA, 2008). Hawassa is frequently visited by national and international tourists. The municipality of Hawassa town has its abattoir that gives service to the town's community.

On average, 100–150 adult beef cattle are slaughtered per day at Hawassa Municipality abattoir, except on Wednesdays and Fridays. After arriving at the slaughterhouse, the animals are inspected, and those fit to be slaughtered are moved into pens where they spend an average of 8–10 h without feed before slaughter.

2.2. Sampling and sample collection

The study involved bacteriological examination of samples collected from 150 apparently healthy slaughtered cattle, 11 slaughterhouse floor swabs (SHFS) and 100 beef samples from butcher shops for *Salmonella*. The sample size for the slaughter cattle was calculated with an expected 7% estimated prevalence (Alemu and Zewde, 2012), desired 95% confidence interval and 5% precision (Thrusfield, 2005) which gives a sample size of 100 animals; the sample size was increased to 150 to improve precision. The study animals were selected with systematic random sampling technique from a batch of cattle destined to be slaughtered on the sample collection day. On each sampling day, which was held weekly, four types of samples were collected from 10 to 15 selected cattle. The chosen animals were followed along the slaughtering process to collect the appropriate samples. Accordingly, carcass swabs (CS), mesenteric lymph nodes (MLN), spleen and cecal content (CC) samples, 150 each, were collected aseptically in separate sterile containers making the total number of samples collected from slaughtered animals 600. Simultaneously, one weekly swab sample was collected for a total of 11 weeks (representing sample collection dates) from slaughterhouse floor on the same date of sample collection from slaughtered animals by rubbing a 1 m*2 m (2 m^2) floor area using a gauze swab. Additionally, 100 beef samples were collected from 100 randomly selected butcher shops in the town. The butcher shops were selected from a list of butcher shops registered in the town with a simple random sampling technique. A total of 711 samples from various sources were collected during the study, for isolation and characterization of *Salmonella*.

For CS sample, sterile gauze moistened with buffered peptone water (BPW) was rubbed over different parts of the carcass (100 cm^2 area each on the rump, flank, brisket and neck) and was then placed into wide-mouthed, screw-capped, 30 ml, universal glass bottles. Cecal content samples were collected directly from the cecum into sterile universal bottles through a puncture made with sterile scalpel blades. The samples were transported to the Microbiology Laboratory of the School of Veterinary Medicine of Hawassa University in ice boxes with ice packs. Upon arrival at the laboratory the samples were stored at 4 °C in the refrigerator for immediate microbiological analysis within 24 h of sampling. Samples were identified by date of collection, sources and sample type.

2.3. Isolation and identification

For the isolation and identification of *Salmonella*, the technique recommended by the International Organization for Standardization (ISO-6579) (ISO, 2002) and Quinn et al. (1999) were employed as previously described (Aragaw et al., 2007) with minor modification. For pre-enrichment, 25 g of MLN, spleen and meat samples each were weighed, passed over flame to disinfect the surface (MLN and spleen), cut into small pieces on sterile Petri dish using sterile scalpel blade and placed in screw capped wide mouth bottles containing 225 ml buffered peptone water (BPW) (HiMedia, Mumbai, India), thoroughly mixed by shaking and incubated for 16–20 h at 37 °C. Similarly, 25 g of CC was placed in a wide mouth container containing 225 ml of BPW shaken and incubated as described above. The swab samples (CS and SHFS) were also pre-enriched in 225 ml BPW. Rappaport-Vassiliadis (RV) medium (HiMedia, Mumbai, India) was used for selective enrichment of the samples. After vortexing the pre-enrichment broth culture 0.1 ml was transferred to a tube containing 10 ml of RV medium and incubated at 42 °C for 24 h.

Xylose lysine desoxycholate (XLD) agar and Salmonella-Shigella (SS) agar (both from Oxoid, Hampshire, England) plates were used for plating out and identification. A loop-full of inoculum from RV broth culture was plated onto XLD and SS plates each and incubated at 37 °C for 24 h. After incubation, the plates were examined for the presence of typical and suspect colonies. Up to five of these colonies were selected from the selective plating media, streaked onto the surface of nutrient agar (HiMedia, Mumbai, India) plates and incubated at 37 °C for 24 h. Presumptive *Salmonella* colonies were characterized using biochemical tests following standard methods (Quinn et al., 1999; ISO, 2002).

Biochemically typical *Salmonella* isolates were stab-cultured in tryptone soya agar (Titan Biotech Ltd., Rajasthan, India) and shipped to the Public Health Agency of Canada, Office International des Epizooties (OIE) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada for serotyping and phage typing.

2.4. Serotyping and phage typing

For serotyping, the somatic (O) antigens were determined by slide agglutination tests (Ewing, 1986) and flagellar antigens were determined using a microplate agglutination technique (Shipp and Rowe, 1980). The antigenic formulae of Grimont and Weill (2007) were used to identify and assign the serotypes of the isolates.

Phage typing of *S. enterica* serotype 1:4,5,12:i:- isolates was performed by the methods developed by Callow (1959) and extended by Anderson et al. (1977) with reference phages obtained from the Public Health England, Gastrointestinal Bacteria Reference Unit, Colindale, England and the Public Health Agency of Canada, National Laboratory for Enteric Pathogens, Winnipeg, Canada.

Table 1
Resistance breakpoints for a panel of antimicrobials used to determine antimicrobial susceptibility among *Salmonella* isolates.

Antimicrobial	Range (μg/ml)	Susceptible (μg/ml)	Resistant (μg/ml)	Reference
Amoxicillin/Clavulanic Acid (AMC)	1–32	< = 8	> = 32	CLSI M100-S24[a]
Ampicillin (AMP)	1–32	< = 8	> = 32	CLSI M100-S24[a]
Azithromycin (AZM)	0.12–16	< = 16	> = 32	No CLSI Interpretive Criteria[b]
Cefoxitin (FOX)	0.5–32	< = 8	> = 32	CLSI M100-S24[a]
Ceftiofur (TIO)	0.12–8	< = 2	> = 8	CLSI VET01-S2[c]
Ceftriaxone (CRO)	0.25–64	< = 1	> = 4	CLSI M100-S24[a]
Chloramphenicol (CHL)	2–32	< = 8	> = 32	CLSI M100-S24[a]
Ciprofloxacin (CIP)	0.015–4	< = 0.06	> = 1	CLSI M100-S24[a]
Gentamicin (GEN)	0.25–16	< = 4	> = 16	CLSI M100-S24[a]
Nalidixic Acid (NAL)	0.5–32	< = 16	> = 32	CLSI M100-S24[a]
Streptomycin (STR)	2–64	< = 32	> = 64	No CLSI Interpretive Criteria[b]
Sulfisoxazole (SOX)	16–256	< = 256	> = 512	CLSI M100-S24[a]
Tetracycline (TCY)	4–32	< = 4	> = 16	CLSI M100-S24[a]
Trimethoprim/Sulphamethoxazole (SXT)	0.12–4	< = 2	> = 4	CLSI M100-S24[a]

[a] CLSI M100-S24.
[b] No CLSI breakpoints for *Enterobacteriaceae* available for this antimicrobial. Breakpoints based on distribution of MIC's and were harmonized with NARMS.
[c] CLSI VET01-S2.

2.5. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was carried out at Public Health Agency of Canada, National Microbiology Laboratory at Guelph, Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), Guelph AMR Testing Laboratory, Guelph, Ontario, Canada. All the 14 isolates, obtained during the study, were tested for a panel of 14 antimicrobials (Panel Type: CMV3AGNF) using the Sensititre® Automated Microbiology System (Trek Diagnostics, Inc., Weslake, OH, USA) microtitre plates to determine the minimal inhibitory concentration (MIC) as per the manufacturer's instructions. Antimicrobial susceptibility results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) (2013, 2014) guideline. The list of a panel of antimicrobials utilized, their symbols, and concentrations and breakpoints are shown in Table 1.

2.6. Data management and analysis

The data collected for each slaughtered animal included sex, age, breed, body condition, origin and results of the bacteriological examination of the samples as *Salmonella* negative or positive. An animal was considered *Salmonella* positive when it was positive for at least one of CC, MLN or spleen samples. Carcass swab sample was used as a measure of contamination. The data were entered in Microsoft Excel and were analyzed with Stata statistical software version 13 (Stata Corp., College Station, TX).

3. Results

3.1. Occurrence of Salmonella

Out of the total of 711 samples examined 14 (2.0%, 95% CI: 1.2, 3.3) were found positive for *Salmonella*. At an animal level (if *Salmonella* was isolated from at least one of the CC, MLN or spleen samples) *Salmonella* was isolated from four (2.7%, 95% CI: 1.0, 7.0) of the 150 slaughtered cattle examined. Two (1.3%, 95% CI: 0.3, 5.3) of the 150 carcasses were found contaminated with *Salmonella*. The occurrence at sample level for the slaughtered cattle was 2.7% (95% CI: 1.0, 7.0) for CC and 1.3% (95% CI: 0.3, 5.3) for MLN. Four of the 100 beef samples (4.0%, 95% CI: 1.5, 10.3) collected from butcher shops and two of the 11 (18.2%, 95% CI: 3.5, 58.0) SHFS samples yielded *Salmonella* (Table 2).

Table 2
Occurrence of *Salmonella* by sample type.

Sample type	Number of samples		Proportion (%)	95% CI
	Examined	Positive		
CC	150	4	2.7	1.0–7.0
CS	150	2	1.3	0.3–5.3
MLN	150	2	1.3	0.3–5.3
Spleen	150	0	0	
SHFS	11	2	18	3.5–58
Beef	100	4	4.0	1.5–10.3
Total	711	14	2.0	1.2–3.3

CC, cecal content; CS, carcass swab; MLN, mesenteric lymph nodes; SHFS, slaughterhouse floor swab.

3.2. Serotype distribution

A total of 14 isolates belonging to the *Salmonella enterica* subsp. *enterica* group were recovered during the study. These isolates belonged to 3 serotypes namely *Salmonella enterica* serotype Muenchen (4 isolates), *S. enterica* serotype Korovi (5) and *S. enterica* serotype 1,4,5,12,i:- (5). All of the *S. enterica* serotype 1:4,5,12:i:- isolates were found to belong to phage type 120.

Salmonella enterica serotype Korovi was isolated from CC (n = 3), CS (1) and SHFS (1), and *S. enterica* serotype 1:4,5,12:i:- was isolated from CC (n = 1), MLN (2), CS (1) and SHFS (1). *Salmonella enterica* serotype Muenchen, however, was isolated only from beef samples collected from butcher shops (Table 3).

Only one individual animal was detected having two different serotypes in various samples: *S. enterica* serotype Korovi *and S. enterica* serotype 1,4,5,12:i:- in CC and MLN respectively.

3.3. Antimicrobial resistance

All the 14 isolates of *Salmonella* obtained in the study were tested for susceptibility to 14 antimicrobials (Table 1) and all of them were found susceptible to all of the antimicrobials used.

4. Discussion

The occurrence of *Salmonella* in slaughtered cattle, CS and retail beef observed in this study was generally low compared to previous reports from different parts of the county (Ashenafi, 1994; Nyeleti et al., 2000; Alemayehu et al., 2003; Sibhat et al., 2011; Alemu and Zewde, 2012; Garedew et al., 2015). However, we acknowledge that our

Table 3
Serotype distribution of *Salmonella* isolated from slaughtered cattle, SHFS and retail beef with sample type.

Sample type	No. examined	Serotype	No. of isolates	*Salmonella* occurrence
CC	150	*S.* Korovi	3	2.7%
		S. 1:4,5,12:i:-	1	
MLN	150	*S.* 1:4,5,12:i:-	2	1.3%
Spleen	150	–	0	0%
CS	150	*S.* Korovi	1	1.3%
		S. 1:4,5,12:i:-	1	
Beef	100	*S.* Muenchen	4	4.0%
SHFS	11	*S.* Korovi	1	18%
		S. 1:4,5,12:i:-	1	
Total	711		14	2.0%

CC, cecal content; MLN, mesenteric lymph nodes; CS, carcass swab; SHFS, slaughterhouse floor swab.

carcass sample size was too small to identify any trends and possible comparison with other studies. The reason for this was that the sample size formula used was not adjusted for precision down form the default 5% used. Thus, a more proper precision of 7% ± 2% would have given a sample size of 625.

Salmonella occurrence in slaughtered animals, tissues and carcasses may be affected by hygienic conditions of holding pens, transportation stress, length of stay in lairage, hygienic status of the slaughterhouse environment and even the prevalence of *Salmonella* in beef cattle population supplying the slaughterhouses (Smeltzer et al., 1980; Morgan et al., 1987; Berends et al., 1997; Mannion et al., 2012).

We isolated three serotypes of *Salmonella* belonging to the *S. enterica* subsp. *enterica* group. Both *Salmonella enterica* serotype Korovi and serotype 1,4,5,12:i:- were isolated from CC, CS and slaughterhouse floor, while serotype 1,4,5,12:i:- and serotype Muenchen were isolated only from MLN and beef, respectively. Earlier works in Ethiopia have reported the isolation of *S. enterica* serotype Muenchen from cattle (Nyeleti et al., 2000), camels (Pegram et al., 1981; Molla et al., 2004) and pigs (Aragaw et al., 2007). The serotype was among the most frequently isolated serotypes from meat or poultry in the US (FSIS, 2016) and the ten most commonly identified serotypes causing human salmonellosis in 2012 in the US (CDC, 2014). *Salmonella enterica* serotype Muenchen has been associated with several disease outbreaks in people in different parts of the world (Proctor et al., 2001; Buchholz et al., 2005; OzFoodNet, 2005; CDC, 2016).

Salmonella enterica serotype 1,4,5,12:i:- and serotype Korovi, the other two serotypes isolated in the present study, are to the best of our knowledge reported here for the first time in Ethiopia, while none of the common previously reported serotypes in cattle (Alemayehu et al., 2003; Sibhat et al., 2011; Alemu and Zewde, 2012) were detected. However, the isolation of *S. enterica* serotype 1,4,5,12:i:- is in agreement with the rise of the serotype throughout the world. The recently emerged serotype has become one of the most frequently isolated serotypes in many countries (Echeita et al., 1999, 2001; CDC, 2008; Switt et al., 2009; Hauser et al., 2010; Mandilara et al., 2013; FSIS, 2016; Yang et al., 2015). Several molecular studies conducted on this serotype in different parts of the world (Echeita et al., 2001; Zamperini et al., 2007; Bugarel et al., 2012; Mandilara et al., 2013; Yang et al., 2015) demonstrated that it is a monophasic variant of *S. enterica* serotype Typhimurium unable to express the phase 2 flagellar antigens (lacking the second phase of H-antigen).

During 2011 *Salmonella enterica* serotype 1,4,5,12:i:- was the third frequently isolated serotype in Ireland next only to *S. enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis (NSSLRL, 2011). During 2011 and 2012, it was the 3rd most frequently reported serotype from confirmed human cases in the EU/EEA countries (EFSA/ECDC, 2014a). It was also the 5th most commonly isolated serotype among laboratory-confirmed human salmonellosis infections in the US in 2012 (CDC, 2014). This bacterium has also ranked among the four most frequent serotypes causing human salmonellosis in China (Yang et al., 2015). The identification of this serotype, which emerged in Europe in the mid-1990s and growing fast in proportional prevalence, in Ethiopia indicates that the serotype might have been imported to the country through contaminated foods or infected animals or human beings. The antimicrobial profiles of our *S. enterica* serotype 1,4,5,12:i:- isolates, however, differ from the most commonly encountered multidrug-resistant isolates (clones) reported elsewhere (CDC, 2008; EFSA/ECDC, 2014b; Yang et al., 2015).

Although many studies consider pigs as the reservoir of *S. enterica* serotype 1,4,5,12:i:- (De la Torre et al., 2003; Hauser et al., 2010), outbreaks associated with beef have been reported in Denmark (ECDC, 2012). Beef, along with pork, was reported to be one of the most important reservoirs of this serotype in China (Yang et al., 2015).

There are only a few published reports of isolation of *S. enterica* serotype Korovi from around the world. It was first reported from a serpent from the then Belgian Congo in 1955 (Kauffmann and van Oye, 1955). It was also isolated from 'lizards and snakes' in Sweden in 1994 for the first time in that country (only one isolate) (Boqvist et al., 2003). Our findings are the first report of the serotype from Ethiopia. The isolation of this rare serotype, which has been reported mainly from reptiles, in a relatively high frequency (n = 3) in the CC of slaughter cattle on different sample collection dates and its detection from CS and slaughterhouse floor samples needs further study.

Contrary to our finding of pan-susceptibility in all 14 isolates obtained in our study, *S. enterica* serotype 1,4, [5],12:i:- was reported to be characterized by multidrug resistance (; Echeita et al., 1999, 2001; Mandilara et al., 2013; EFSA/ECDC, 2014b, Yang et al., 2015). Extremely high levels of resistance were observed for ampicillin, streptomycin, sulfonamides and tetracycline (resistance [R] type-ASSuT) in EU (EFSA/ECDC, 2014b) and China (Yang et al., 2015). *Salmonella enterica* serotype Muenchen has also been found in some studies to demonstrate multiple drug resistance for up to seven antimicrobials: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, amoxicillin/clavulanic acid, and kanamycin (R-type ACSSuT-AxK) (Gebreyes and Thakur, 2005). Our finding was, however, consistent with previous reports in Ethiopia which indicated susceptibility of the serotype to all antimicrobials tested (Molla et al., 2004; Aragaw, 2005). As the lack of antimicrobial resistance of the isolates observed in this study was based on a small number of observations (n = 14), there is a need for a study with larger number of isolates to substantiate it.

The results of this study indicate that the occurrence of *Salmonella* in cattle slaughtered at Hawassa and beef sold in butcher shops in the town are relatively low, with 2.3% slaughter cattle and 4.0% fresh retail beef positive for culture. The study also demonstrated that serotypes which are characterized by multiple drug resistance somewhere may be pan-susceptible elsewhere. This study contributes to the global information on the occurrence and antibiogram of *Salmonella* in general and the expanding *S. enterica* serotype 1,4,5,12:i:- and the rare and usually reptile-associated *S. enterica* serotype Korovi in particular as the first report of their isolation in Ethiopia.

The contamination of carcasses and beef with *Salmonella* serotypes known to cause human infections, although at a low level, is a significant public health risk in Ethiopia where a considerable proportion of the population consumes raw or undercooked beef. Efforts to keep the prevalence low through control in animals, prevention of contamination of animal products at slaughter and butcher shops, and adequate cooking of meat are recommended.

Conflict of interest

The authors declare that they have no conflict of interest.

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