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Occurrence and characterization of nontyphoidal *Salmonella* in retail table eggs in Kandy district of Sri Lanka

R.S. Kalupahana [a, *], D.I.G. Rajapaksa [a], P.S. Fernando [b], D.S. Thilakarathne [a], P. Abeynayake [a]

[a] Department of Veterinary Public Health and Pharmacology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka
[b] Veterinary Research Institute, Gannoruwa, Peradeniya, Sri Lanka

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ABSTRACT

Salmonellosis in humans is typically a foodborne bacterial zoonotic disease. In this regard, eggs and egg products contaminated with nontyphoidal *Salmonella* represent a serious threat to consumers. Eggs are one of the most common sources of animal protein for Sri Lankans but minimum attention is paid to their quality assurance. Except for a few packaged eggs sold under brand names for higher prices, in general, grading, cleaning or cooling is not practised before sale posing a microbiological health hazard to the general public. Hence, the objective of this study was to identify the presence of *Salmonella* in raw table eggs available at retail outlets in one selected district of Sri Lanka. Eggs were purchased from 100 retail outlets situated in highly populated areas of the district. Samples were tested for the presence of *Salmonella* in eggshell washings and egg contents, separately. Isolation and identification of *Salmonella* was performed according to standard methods. The isolates were serotyped and their antimicrobial sensitivity was determined. *Salmonella* was isolated from the eggs purchased from 15 retail outlets, of which 12 yielded *Salmonella* from shell washings and three from egg contents. The serovars identified were *S.* Mbandaka, *S.* Braenderup, *S.* Corvallis, and *S.* Emek. Except one isolate showing resistance to nalidixic acid and another to a third generation cephalosporin, other isolates were sensitive for the tested antimicrobials. According to the results of this study, retail raw table eggs can be considered as an important source of nontyphoidal *Salmonella* to egg consumers in Sri Lanka.

1. Introduction

Salmonellosis is an infectious disease of humans and animals caused by the bacteria *Salmonella* (OIE, 2010, Chapter 2. 9. 9). Among more than 2500 *Salmonella* serovars, nontyphoidal salmonellae are important foodborne zoonotic pathogens (Hohmann, 2001). *Salmonella* colonizes in the gastrointestinal tracts of a broad range of animal species. This bacterium can be either host adapted or non-host adapted. The infectious disease in poultry is caused by non motile, typhoidal *Salmonella* serovars: *Salmonella enterica* serovar Pullorum (*S.* Pollorum) and *S. enterica* serovar Gallinarum (*S.* Gallinarum). These serovars are highly adapted to the host with little importance in public health (Shivaprasad, 2000). However, non-host adapted serovars can colonize in many hosts including poultry, cattle and swine, and food derived from these species can be contaminated with the *Salmonella* in any level of farm to fork food supply chain (Forshell & Wierup, 2006).

Comparing to other animal originated food commodities, raw table eggs and egg products are common vehicles for foodborne Salmonellosis in humans (Threlfall et al., 2014). Eggs can be contaminated by *Salmonella* either through vertical transmission in which organism can enter into newly formulating eggs while eggs are being passed through the reproductive tract of the infected birds or by horizontal transmission where faeces, utensils, pests or handlers contaminate surfaces of eggs after the egg is laid (Gast, Guraya, Jones, & Anderson, 2014; Shivaprasad, 2000; Utrarachkij et al., 2012). Although *S. enterica serovar* Enteritidis (*S.* Enteritidis) and *Salmonella eneterica* serovar Typhimurium (*S.* Typhimurium) are common causes for poultry associated foodborne illnesses in human (Galanis et al., 2006; Hendriksen et al. 2011) outbreaks due to other nontyphoidal serovars such as *S.* Mbandaka have also been

* Corresponding author.
E-mail address: ruwanikalupahana@yahoo.com (R.S. Kalupahana).

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reported (Bäumler, Hargis, & Tsolis, 2000; Paine et al., 2014; Scheil, Cameron, Dalton, Murray, & Wilson, 1998). Similarly, recent review by Threlfall and co-workers, using information from published literature and epidemiological databases, has clearly mentioned the importance of serovars other than -*S*. Enteritidis as a cause of egg-associated Salmonellosis in human (Threlfall et al., 2014).

In Sri Lanka, published evidences on presence of *Salmonella* in poultry production chain are very limited. According to a study conducted in our laboratory in the year 2008, by testing 128 broiler meat samples representing four provinces of the country, *Salmonella* contamination was found as 12% (unpublished data). In addition, Liyanagunawardena, et al., 2012 reported that *S*. Pullorum and *S*. Gallinarum, are the commonly associated *Salmonella* serovars in poultry breeder farms in the country. Although many research has been conducted to understand *Salmonella*-contaminated eggs as a source of foodborne Salmonellosis in developed countries, such studies are scarce in Asian countries like Sri Lanka. Nevertheless, eggs are one of the most common sources of animal proteins for Sri Lankans. Current per capita consumption of eggs in the country is 108 (DAPH, 2014). In Sri Lanka, table eggs are commonly available throughout the country. A few brands of cleaned and pre-packaged eggs are available for higher prices in large supermarkets and also in some retail outlets in main cities. Loose eggs without packaging, stored in wooden crates, often with some packing materials such as hay or paddy husk, is the most common form available in retail outlets. Generally, those eggs are not graded or cleaned before sale and often visible dirt can be seen on the shells of eggs.

Dirty eggs can contaminate the food chain at many stages not only while preparing egg products but also due to cross contamination with other food during storage in the refrigerator and processing in the kitchen which pose the highest risk to humans. There can be contamination of hands and surfaces with *Salmonella* and other food borne pathogens during food preparation (Carrasco, Morales-Rueda, & García-Gimeno, 2012; Gorman, Bloomfield, & Adley, 2002; Soares et al., 2012). With the recent trend in fast food systems in the country where food is subjected to less heat and/or prepared in small road side boutiques, the risk due to cross contamination can be significant. Identifying this risk is highly necessary when public health is concerned. In the absence of supporting evidences, such as prevalence of nontyphoidal *Salmonella* in poultry layer farms or epidemiological data on human Salmonellosis outbreaks due to consumption of eggs in Sri Lanka, this study was aimed at isolating, identifying and antimicrobial resistance profiling of *Salmonella* serovars present in retail table eggs.

2. Materials and methods

2.1. Sampling

Based on the current practices in egg production chain and observations made in the retail outlets in the said district, we hypothesised that considerable number of consumers may expose to potentially pathogenic *Salmonella* through raw table eggs. Therefore, it was decided to test table eggs available at 100 randomly selected retail shops for the presence of *Salmonella*.

Kandy district consists of 20 administrative regions called divisional secretariat divisions (DSD) (http://www.kandy.dist.gov.lk). In order to select 100 shops, DSDs were listed considering the human population, from the highest to the lowest, and the top 10 DSDs were selected. Convenience sampling was carried out and randomly selected 10 retail outlets from each DSD, often situated close to centre of the main towns, were sampled. Fresh eggs either in pre-packaged form or loose, were purchased as a normal customer without giving any special instructions to retailer. One pack of pre-packaged, containing 10 eggs or 10 loose eggs per shop were purchased, transported to the laboratory within 2 h of collection. Five randomly selected eggs, out of the 10 purchased, were pooled to make one sample for testing. The study was carried out within a period of one year.

2.2. Isolation of Salmonella

Egg shell washings and egg contents were tested separately. Egg shell washings were obtained by immersing five eggs in 225 ml of sterile buffered peptone water (BPW) (Oxoid, UK). The eggs were kept in BPW for 30 min and rubbed gently through the bag intermittently. Washed eggs were taken out and disinfected using 70% ethanol, broken with a sterile knife and internal contents were collected into a sterile stomacher bag. After mixing the contents thoroughly, 25 ml of egg content was added to 225 ml of BPW.

For isolation of *Salmonella* spp., the standard method described by the International Organization for Standardization (ISO-6579) was used. Briefly, the egg shell washings and internal contents, in separate containers of BPW, were incubated at 37 °C for 24 h. After incubation, samples were selectively enriched in Rappaport Vassiliadis broth at 42 °C for 24 h. Cultures were then streaked onto brilliant green agar (Oxoid, UK) and xylose lysine deoxycholate agar (Oxoid, UK) and incubated at 37 °C for 24 h. The suspected colonies were cultured on nutrient agar and subjected to biochemical tests which included reactions on triple sugar iron agar, citrate, urease, and indole tests. The confirmed isolates as described in Clinical Veterinary Microbiology by (Quinn, Carter, Markey, & Carter, 1994), were sent to an overseas laboratory for serotyping and antimicrobial susceptibility testing.

2.3. Serotyping and antimicrobial susceptibility testing

All the *Salmonella* isolates were serotyped at the WHO (World Health Organization) National Salmonella and Shigella Center, Bangkok, Thailand by slide agglutination. O and H antigens were characterized by agglutination with hyper immune sera (S & A Reagents Laboratory, Ltd., Bangkok, Thailand) and serotypes were assigned based on the Kauffmann-White scheme (Grimont & Weill, 2007).

Antimicrobial sensitivity patterns of the isolates were identified by determining minimum inhibitory concentrations (MIC), at the DTU-Food, Denmark using a commercially prepared, dehydrated panel (Sensititre; TREK Diagnostic Systems Ltd., East Grinstead, England). Since the *Salmonella* isolates were non-clinical samples, epidemiological cut off values were applied according to EUCAST recommendations (www.eucast.org) to determine resistance profiles. The following 12 antimicrobials and cut-off values (mg/L) to determine resistance (R) were used: ampicillin ($R \geq 8$), cefotaxime ($R \geq 0.5$), chloramphenicol ($R \geq 16$), ciprofloxacin ($R \geq 0.064$), colistin ($R \geq 2$), gentamicin ($R \geq 2$), meropenem ($R \geq 0.125$), nalidixic acid ($R \geq 16$), ceftazidime ($R \geq 2$), tetracycline($R \geq 8$), tigecycline ($R \geq 1$), and trimethoprim ($R \geq 2$).

3. Results

The eggs purchased from 100 different retail outlets (10 eggs from each outlet), which included six pre-packaged eggs belonging to different brands and loose eggs, were tested for *Salmonella*, both in egg shell washings and egg contents. Of the 200 analyses carried out 15 became positive for *Salmonella*. Out of 15 samples, 12 yielded *Salmonella* from shell washings and three from egg contents. None of the samples were *Salmonella* positive in both the shell washing and the contents. Accordingly, overall prevalence was 15% (Table 1).

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Table 1
Salmonella serovars isolated from eggs and their sensitivity to selected 12 antimicrobials.

Salmonella serovars	Minimum inhibitory concentrations (mg/L)											
	AMP	FOT	CHL	CIP	COL	GEN	MERO	NAL	TAZ	TET	TGC	TMP
Egg shell washings												
1. *Salmonella* serovar Emek	1	0.25	8	0.06	2	0.5	0.03	4	0.5	2	0.25	0.25
2. *Salmonella* serovar Mbandaka	1	0.25	8	0.015	2	1	0.03	4	0.5	2	0.25	0.25
3. *Salmonella* serovar Corvallis	1	0.25	8	0.5	2	1	0.03	16	0.5	2	0.25	0.25
4. *Salmonella* serovar Mbandaka	1	0.25	8	0.015	2	0.5	0.03	4	0.5	2	0.5	0.5
5. *Salmonella* serovar Corvallis	1	0.25	8	0.015	2	0.5	0.03	4	4	2	0.25	0.25
6. *Salmonella* serovar Corvallis	1	0.25	8	0.5	2	0.5	0.03	8	0.5	2	0.25	0.25
7. *Salmonella* serovar Mbandaka	2	0.25	8	0.015	2	1	0.03	4	0.5	2	0.5	0.5
8. *Salmonella* serovar Braenderup	2	0.25	8	0.015	2	1	0.03	4	0.5	2	0.5	0.5
9. *Salmonella* serovar Mbandaka	2	0.25	8	0.015	2	1	0.03	8	0.5	2	0.5	0.5
10. *Salmonella* serovar Braenderup	1	0.25	8	0.015	2	0.5	0.03	4	0.5	2	0.5	0.5
11. *Salmonella* serovar Braenderup	1	0.25	8	0.015	2	0.5	0.03	4	0.5	2	0.5	0.5
12. *Salmonella* serovar Braenderup	1	0.25	8	0.015	2	0.5	0.03	4	0.5	2	0.5	0.25
Egg contents												
1. *Salmonella* serovar Mbandaka	1	0.25	8	0.015	2	1	0.03	4	0.5	2	0.25	0.25
2. *Salmonella* serovar Mbandaka	1	0.25	8	0.015	2	1	0.03	4	0.5	2	0.25	0.5
3. *Salmonella* serovar Mbandaka	1	0.25	8	0.015	2	1	0.03	4	0.5	2	0.25	0.25

Abbreviations: AMP, ampicillin; FOT, cefotaxime; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; MERO, meropenem; NAL, nalidixic acid; TAZ, ceftazidime; TET, tetracyclin; TGC, tigecycline; TMP, trimethoprim. Underlined values indicate the MICs interpreted as resistant to given antimicrobial. Results were interpreted according to EUCAST guidelines on the basis of epidemiological cut-off values. The epidemiological cut-off values (mg/L): ampicillin ($R \geq 8$), cefotaxime ($R \geq 0.5$), chloramphenicol ($R \geq 16$), ciprofloxacin ($R \geq 0.064$), colistin ($R \geq 2$), gentamicin ($R \geq 2$), meropenem ($R \geq 0.125$), nalidixic acid ($R \geq 16$), ceftazidime ($R \geq 2$), tetracycline($R \geq 8$) , tigecycline ($R \geq 1$), and trimethoprim ($R \geq 2$).

It was noted that, none of the pre-packaged eggs (n = 6) was contaminated with *Salmonella*.

All isolates were distributed among four serotypes, namely *Salmonella* Mbandaka (46%), *Salmonella* Braenderup (26%), *Salmonella* Corvallis (20%), and *Salmonella* Emek (7%). S. Mbandaka, the most commonly found serotype was also the only serotype isolated from egg contents.

As shown in Table 1, MIC determination of the 15 isolates revealed a low level of resistance to the antimicrobials tested. However, two isolates were resistant to either nalidixic acid or ceftazidime.

4. Discussion

This study indicated a noteworthy level of *Salmonella* contamination which was 15% in retail raw table eggs in the studied district of Sri Lanka. Even though this is a novel finding to the country, contamination of raw table eggs with *Salmonella* has been extensively reported elsewhere in the world. In the UK, the reported level of contamination was 0.38% according to a survey carried out in food service premises from 2005 to 2006 (Little et al., 2008). India has reported 7.7% contamination by individually testing 492 eggs collected from retail shops in a residential area (Suresh, Hatha, Sreenivasan, Sangeetha, & Lashmanaperumalsamy, 2006). Examining 100 sales outlets, the contamination level with nontyphoidal *Salmonella* identified in the present investigation was as high as 15%, emphasizing a significant public health hazard that can be due to handling or consuming of those eggs.

In the present study, the level of eggshell contamination exceeded the level of contamination found in egg contents. This implicates a higher risk to public because dirty eggs can contaminate the food chain at many stages as a result of cross contamination.

The excessive surface contamination could well be due to poor hygienic standards currently practised from farms to retail shops during egg production, storage, transportation, and retailing. Cleaning of eggshells and cooling of eggs, before sale, are not generally practised by the average retailers. Additionally, other than plastic crates, wooden crates packed with dry hay, wood shavings, shredded paper, or paddy husks are often used to transport eggs from farm to retail and to store at retail. These packing materials are not cleaned and prepared specifically for transport of eggs. As observed by (Jayasena, Cyril, & Jo, 2012) out of 482 eggs collected from Colombo wholesale egg market, only 60.37% were in the desirable quality based on the specifications recommended by the Sri Lankan Standard Institute (SLS/959:1992) for chicken eggs. However, the local standards do not specify microbiological quality requirements for chicken eggs indicating the poor attention paid for this important parameter. As described by previous studies, maintaining of microbiological quality in eggs is important. For instance, mesophilic aerobic bacteria can survive on eggshells regardless of storage conditions up to 21 days. Further, the survival rate of *S. enterica* on egg shells can be increased with the presence of chicken faeces (Park et al., 2015). In order to reduce the risk of microbiological hazards, cooling of eggs has been recommended (Gross et al., 2015; Olivier et al., 2009). Such data is not available in Sri Lanka and refrigeration is not a mandatory requirement. Therefore, it is possible that the uncontrolled marketing system existing in the country contributes to the risk of eggshell contamination with microorganisms including *Salmonella*.

Additionally, microbial contamination of eggs could be due to infected birds at the farm level. *S.* Pullorum and *S.* Gallinarum are known to be common causes of poultry diseases leading to economic losses- in the country (Priyantha, 2009; Priyantha, Vipulasiri, & Gunawardana, 2012). In Sri Lanka, except few farms where cage system of layer management is practised, the most common method of rearing layers is deep litter open house system. In this system, with less bio-security, birds are in contact with the natural environment resulting higher risk for *Salmonella* contamination. According to studies done elsewhere, eggs produced by layers can be contaminated with *Salmonella* regardless of the management system at varying degrees (Parisi, Northcutt, Smith, Steinberg, & Dawson, 2015).Currently, there is no published evidence on prevalence of nontyphoidal *Salmonella* in layer flocks in Sri Lanka but this information is needed to rule out the contribution of live poultry to egg contamination.

Serotyping results of the present study revealed the absence of *S.* Enteritidis and *S.* Typhimurium in raw table eggs in Kandy district, though these serovars has been reported by other local studies. The study done by Weerasooriya et al. found that *S.*

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Typhimurium in a poultry processing plant in Kandy district (Weerasooriya, Kalupahana, & Abeynayake, 2008) while a study by Wijemanne reported *S.* Enteritidis in a poultry breeder farm (Wijemanna, 2008). The absence of *S.* Pullorum and *S.* Gallinarum, the commonly reported poultry specific serovars in the country, was another significant finding of this study. Absence of those serovars may possibly be due to the strict *Salmonella* control programme implemented in breeder farms and recently introduced vaccination programmes for commercial layer flocks. Under this vaccination programme, breeder farms and commercial layer farms are allowed to use SG 9R live vaccine and *Salmonella* killed vaccine, respectively. The killed vaccine containing *S.* Enteritidis and *S.* Typhimurium and live vaccine with *S.* Gallinarum has been shown to exert cross protection (Van Immerseel et al., 2005).

However, the alarming finding was that all the serovars isolated in this study were motile and recorded human pathogens. The predominant serovar identified was *S.* Mbandaka, which has been responsible for human disease outbreaks (Paine et al., 2014; Scheil et al., 1998).There are many reports where *S.* Mbandaka has been isolated as contaminants in table eggs (Martelli & Davies, 2012). In this study, *S.* Mbandaka was isolated from eggshell washings as well as from the content. It was the only serovar isolated from egg content but the shell washings of same samples did not yield *S.* Mabandaka or any other *Salmonella* suggesting the possibility of contamination during the formation of eggs due to infection in layers. This notion cannot be confirmed as there is no published evidence on the occurrence of *S.* Mbandaka in poultry in Sri Lanka. However, Hayward et al. from the United Kingdom have reported possible association of *S.* Mbandaka with chicken (Hayward, Jansen, & Woodward, 2013).

Other serovars isolated in this study, *S.* Braenderup, *S.* Corvallis, and *S.* Emek have also been linked to human disease in other parts of the world (Gupta et al., 2007; Nakao et al., 2015). One published study in Sri Lanka on four human clinical cases due to nontyphoidal *Salmonella* causing bacteraemia has identified *S.* Enteritidis and *S.* Corvallis as the causative agents. In that study all cases had been treated successfully with third generation cephalosporins indicating the importance of antimicrobials in life saving situations (Mubarak & Chandrasiri, 2013). In this context, it is disturbing to note that two *Salmonella* isolates identified in the present study were resistant against important antimicrobials including a third generation cephalosporin. Additionally, the emergence of *Salmonella* species that are resistant to third generation cephalosporins is already a concern elsewhere (Acheson & Hohmann, 2001; Fey et al., 2000).

In Sri Lanka, since there is no active surveillance system to monitor foodborne infections and associated antimicrobial resistance, the full burden of illness due to Salmonellosis and source attribution of human Salmonellosis is presently unknown. However, with 15% of retail outlets having table eggs contaminated with human pathogenic nontyphoidal *Salmonella* is a serious concern. Some isolates showing resistance to important antibiotics further aggravate the problem. Hence, there is a necessity to take measures to prevent *Salmonella* contamination of table eggs. In order to facilitate implementation of proper control measures, identification of the sources of *Salmonella* in retail table eggs by furthering studies is a timely effort. Until such time to minimize public health hazards, consumers should be educated about risks associated with improper handling and consumption of table eggs. The data generated in this study can be utilized to increase vigilance of consumers, poultry producers, public health policy makers, and other stakeholders.

5. Conclusions

Out of the 100 retail outlets, 15 had table eggs contaminated with nontyphoidal *Salmonella* serovars indicating a potential health risk to public. Contamination of eggshell washings was three times higher than that of the egg contents. The serovars identified were *S.* Mbandaka, *S.* Braenderup, *S.* Corvallis and *S.* Emek. Occurrence of these serovars raises concern because of their association with reported human illnesses. Except two isolates, one resistant to nalidixic acid and the other to ceftazidime others were sensitive to all the antimicrobials tested. Assurance of microbiological safety of table eggs through implementation of food safety regulations is necessary to ensure consumer safety.

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