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Emergence and clonal dissemination of *Salmonella enterica* serovar Enteritidis causing salmonellosis in Mauritius

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Abstract

Introduction: For decades, *Salmonella enterica* serovar Enteritidis has been among the most prevalent serovars reported worldwide. However, it was rarely encountered in Mauritius until 2007; since then the number of non-typhoidal *Salmonella* serogroup O:9 (including serovar Enteritidis) increased. A study was conducted to investigate the genetic relatedness between *S. Enteritidis* isolates recovered in Mauritius from food and clinical specimens (stool, blood, and exudate).

Methodology: Forty-seven isolates of *S. Enteritidis* obtained in 2009 from human stools, blood cultures and exudates, and from food specimens were characterized by antimicrobial susceptibility testing and Multiple-Locus Variable-number tandem repeat Analysis (MLVA).

Results: With the exception of a single isolate which demonstrated intermediate susceptibility to streptomycin, all isolates were pansusceptible to the 14 antimicrobials tested. Thirty seven out of the 47 isolates (78.7%) exhibited an indistinguishable MLVA profile which included isolates from ready-to-eat food products, chicken, and human clinical isolates from stool, blood and exudate.

Conclusions: The presence of highly related strains in both humans and raw chicken, and the failure to isolate the serovar from other foods, suggests that poultry is the main reservoir of *S. Enteritidis* in Mauritius and that the majority of human cases are associated with chicken consumption which originated from one major producer. Stool isolates were indistinguishable or closely related to blood and exudate isolates, indicating that, besides gastroenteritis, the same strain caused invasive infections. Control of *S. Enteritidis* by poultry breeders would lower the financial burden associated with morbidity in humans caused by this organism in Mauritius.

Key words: *Salmonella* Enteritidis; antimicrobial resistance; human infections; MLVA; Mauritius

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Introduction

Non-typhoidal salmonellae (NTS) are among the most commonly reported causes of bacterial foodborne illness worldwide [1-3]. In Mauritius, an upper middle income island nation of 1.25 million inhabitants in the southwest Indian Ocean, *Salmonella enterica* has been the bacterial pathogen most frequently isolated from stools of patients for the last 12 years [4,5] and *Salmonella* serovar Typhimurium (*S. Typhimurium*) has been the most commonly encountered serotype; 45% of isolates serogrouped between 1998 and 2009 at the Mauritius Central Health Laboratory (CHL) were O:4, the serogroup of *S. Typhimurium*.

S. Enteritidis is consistently among the two most common serovars reported from clinical specimens

worldwide [6]. In Mauritius, serogrouping is typically performed in lieu of full serotyping. With the exception of one blood isolate in 2006 of non-typhoidal *Salmonella* serogroup O:9, which could have been *S. Enteritidis*, *Salmonella* Enteritidis was not isolated from stool or blood samples collected between 1999 and 2006 in Mauritius. In 2007, non-typhoidal *Salmonella* serogroup O:9 was isolated from two specimens and subsequently, its prevalence increased tremendously in 2008 to become the leading serogroup among stools and blood isolates (CHL data).

Since 2008, non-typhoidal *Salmonella* serogroup O:9 has been isolated in several instances in pure growth from human soft tissue and internal organ

abscesses at the CHL in Mauritius and a total of 33 cases of non-typhoidal *Salmonella* serogroup O:9 bacteraemia have been reported in 2008 and 2009 as shown in Figure 1. *Salmonella* Enteritidis has also been isolated at the CHL since 2008 from several specimens of raw chicken and ready-to-eat food in Mauritius and has been the cause of many foodborne disease outbreaks notified to public health services. However, little is known about the local epidemiology of this serotype.

The objective of the present study was to investigate genetic relatedness among *S. Enteritidis* isolates recovered from food and clinical samples associated with gastroenteritis, bacteremia and other extraintestinal infections in patients from Mauritius. By identifying potential reservoirs of *S. Enteritidis*, this study was also intended to find biological and epidemiological evidence of the probable source of these infections and identify potential interventions aimed at reducing morbidity and mortality caused by *S. Enteritidis* in Mauritius.

Methodology

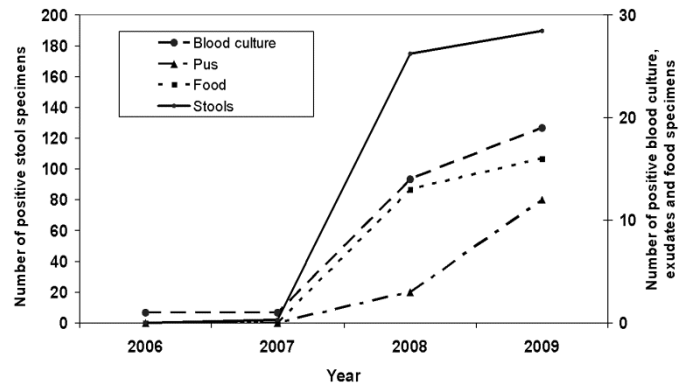
Isolation of *Salmonella* from different sources

The CHL processes all clinical samples sent for bacteriologic investigations from all government healthcare institutions in Mauritius, as well as samples from some private medical clinics. Additionally, the CHL also processes all food samples for microbiologic testing collected by Ministry of Health inspectors during routine inspections or as part of the investigation of reported cases of foodborne illness.

All stool specimens were cultured directly onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Basingstoke, UK) and enriched in selenite enrichment broth (Oxoid, Basingstoke, UK) followed by subculture on XLD agar. Agar plates and broth were both incubated at 37°C for 18 hours. Blood cultures were processed manually by standard procedures [7]. *Salmonella* in raw and in ready-to-eat food specimens was detected by standard methods [8] and identified using biochemical tests [9]. Serogrouping was performed by agglutination with monovalent O antisera (Mast, Bootle, United Kingdom; Remel, Dartford, United Kingdom).

Laboratory registers of stool specimens received in 2009 were reviewed for *Salmonella*-positive cases. Duplicate isolates from the same patient, and isolates from specimens sent for screening purposes were excluded. Additionally, specimen registers of blood and exudates cultured in 2009 were searched for *Salmonella*. Records of the microbiological testing of

Figure 1. Number of *Salmonella* serogroup O:9 isolated from different sources at the Central Health Laboratory, Mauritius; 1998-2009



food specimens performed in 2009 at the CHL were also reviewed for foodstuffs positive for *Salmonella*. Records for 2006, 2007 and 2008 were also examined for trends in the isolation of *Salmonella*.

Selection of isolates

Forty-seven isolates of *Salmonella* serogroup O:9 isolated during 2009, all suspected to be *S. Enteritidis* were selected and sent to the National Institute of Health, Thailand for full serotyping, National Food Institute (DTU-FOOD), Denmark, for minimum inhibitory concentration (MIC) determination, and the Centers for Disease Control and Prevention (CDC), United States of America for Multiple-Locus Variable-number tandem repeat Analysis (MLVA).

The isolates originated from food ($n = 16$), blood culture ($n = 9$), abscesses ($n = 4$) and stool ($n = 18$) specimens (details in Table 1). At the CHL, all *Salmonella* isolates recovered from food are routinely saved; however clinical isolates are only saved if further studies are contemplated. In 2009, an increase in the burden of salmonellosis due to *S. Enteritidis* with a potential link to food was noted and as such, all clinical isolates from November and December 2009 were saved. Additionally, five blood culture isolates from January 2009 were available and were included to obtain information on possible temporal changes in strains causing infections. Isolates were selected based on availability and to ensure representation of food, stool, blood, and exudate isolates. Additionally, stool isolates were selected to ensure a mixture of both sporadic cases and outbreak cases.

Table 1. Meta data of the 47 isolates of *Salmonella* serovar Enteritidis

Isolate number	Date	Origin	Specimen description	Age	Sex	Other information
7033	02.01.09	Human	Blood	24	M	Tourist; Hepatitis C positive
7106	05.01.09	Human	Blood	15	M	On corticosteroids
7310	11.01.09	Human	Blood	36	M	Alcoholic patient
7470	16.01.09	Human	Blood	68	M	Diabetic
7575	20.01.09	Human	Blood	54	F	Neutropenia of unknown cause
421	14.04.09	Food	Mayonnaise	N/A		Food poisoning outbreak (L)
1280	29.10.09	Food	Raw chicken	N/A		Brand P
1346	04.11.09	Food	Raw chicken	N/A		Brand P
1372	12.11.09	Food	Cream cake	N/A		Served at birthday outbreak (M)
8128	13.11.09	Human	Stools	50	F	Sporadic gastroenteritis
8167	16.11.09	Human	Stools	31	F	Sporadic gastroenteritis
8209	20.11.09	Human	Stools	4	F	Sporadic gastroenteritis
8218	21.11.09	Human	Stools	2	M	Sporadic gastroenteritis
11/3627	23.11.09	Human	Pus	43	M	Parotid gland abscess
7597	24.11.09	Human	Blood	7m	M	Acute gastroenteritis
1420	24.11.09	Food	Eclair cream cake	N/A		From bakery (M)
11/9353	25.11.09	Human	Pus	11/2	M	Elbow abscess
7694	26.11.09	Human	Blood	83	M	Chronic renal failure
1445	26.11.09	Food	Raw chicken	N/A		Unknown breeder
8292	27.11.09	Human	Stools	5	F	Family outbreak
1454	30.11.09	Food	Raw Chicken	N/A		Brand P
1463	01.12.09	Food	Raw Chicken	N/A		Brand C
8020	01.12.09	Human	Stools	12	F	Family outbreak
8029	02.12.09	Human	Stools	9m	F	Sporadic gastroenteritis
7083	03.12.09	Human	Blood	21	M	Alcoholic
8069	06.12.09	Human	Stools	11	M	Sporadic gastroenteritis
12/3188	07.12.09	Human	Pus	54	M	Brain abscess; alcoholic
8123	11.12.09	Human	Stools	10	M	Sporadic gastroenteritis
12/9204	15.12.09	Human	Pus	57	F	Neck abscess
8153	16.12.09	Human	Stools	27	M	Food poisoning outbreak (S)
7528	19.12.09	Human	Blood	18	M	Leukaemia patient
1524	21.12.09	Food	Mayonnaise	N/A		Food poisoning outbreak (X)
1525	21.12.09	Food	Chicken kebab in bread	N/A		Food poisoning outbreak (X)
1526	21.12.09	Food	Raw chicken	N/A		From catering premises outbreak (S)
1527	21.12.09	Food	Mayonnaise	N/A		Food poisoning outbreak (S)
1610	24.12.09	Food	Chilli sauce	N/A		Food poisoning outbreak (F)
1611	24.12.09	Food	Uncooked chicken tikka	N/A		Food poisoning outbreak (F)
1614	24.12.09	Food	Raw chicken	N/A		Food poisoning outbreak (F)
8248	24.12.09	Human	Stools	5	F	Food poisoning outbreak (F)
8251	24.12.09	Human	Stools	87	F	Food poisoning outbreak (F)
8268	25.12.09	Human	Stools	14	M	Food poisoning outbreak (F)
8301	26.12.09	Human	Stools	30	M	Food poisoning outbreak (F)
8305	26.12.09	Human	Stools	31	M	Food poisoning outbreak (F)
8310	26.12.09	Human	Stools	43	F	Asymptomatic foodhandler (F)
8316	26.12.09	Human	Stools	35	F	Asymptomatic foodhandler (F)
8319	28.12.09	Human	Stools	11	F	Food poisoning outbreak (F)
1687	29.12.09	Food	Raw chicken	N/A		From breeder which produces brand P

N/A: not applicable; m: month; L, F, M, S and X refer to food poisoning outbreaks location; outbreaks L and S were associated with the same snack bar. P and C refer to two common brands of chicken produced in Mauritius.

Epidemiological data of selected isolates

Epidemiological data on the isolates are summarized in Table 1. Stool isolates #8248, #8251, #8268, #8301, #8305 and #8319 were obtained from patients affected in a large outbreak that resulted in 700 persons seeking medical care and 105 requiring admission. It was linked to consumption of chicken tikka or kebab with home-made mayonnaise and chili sauce bought at a twice-weekly village market. Isolates #8310 and #8316 were recovered in the context of the outbreak investigation from stools of asymptomatic food handlers. Food isolates #1610, #1611 and #1614 were obtained from samples collected at the premises where the chicken were marinated and the home-made mayonnaise and chilli sauce prepared. The caterer reported that the chicken used originated from the same major poultry breeder which markets the raw chicken from which isolate #1687 was subsequently obtained. Moreover, isolates #1280, #1346 and #1454 had previously been found in a brand of raw chicken produced by the same breeder. In contrast, isolate #1463 was found in a brand of chicken produced by a different major poultry breeder and isolate #1445 was obtained from chicken claimed to have been bought at a poultry shop which is supplied by an unspecified small breeder.

Food isolates #1526 and #1527 were obtained from the premises of a snack bar where chicken kebab that was associated with a small outbreak of salmonellosis was prepared; isolate #8153 was from the stools of a patient who became ill in this outbreak. Isolate #421 had previously been found in mayonnaise from the same snack bar following investigation of another small outbreak though no positive human specimen was obtained in that instance. Following an outbreak at a birthday party, food isolate #1372 was found in one of the cream cakes that were served and subsequently isolate #1420 was obtained from an éclair cream cake that was prepared in the bakery which supplied the cakes. Isolates #8292 and #8020 were obtained from two siblings who were admitted to hospital with food poisoning. Food isolates #1524 and #1525 were found in specimens collected at a food outlet following complaints by people who became ill after eating food sold there.

Serotyping

Isolates were serotyped using slide agglutination with hyperimmune sera (S & A reagents lab, Ltd, Bangkok, Thailand) characterizing the O and H antigens. The serotypes were assigned according to the Kauffmann-White scheme [10]. At CDC, the serotype

was confirmed by PCR amplification of the *Sdf I* gene specific for *Salmonella* serovar Enteritidis [11].

Antimicrobial susceptibility testing

Susceptibility to 17 different antimicrobial agents was performed by MIC determination at DTU-Food, Denmark using a commercially prepared, panel of dehydrated antimicrobials (Sensititre; TREK Diagnostic Systems Ltd., East Grinstead, England). [12]. Results were interpreted using EUCAST-recommended cutoff values accessed at <http://www.eucast.org>.

Multiple-Locus Variable-number tandem repeat Analysis

MLVA was performed by following the standardized PulseNet USA protocol for *S. enterica* serotype Enteritidis (Laboratory standard operating procedure for PulseNet MLVA of *S. enterica* serotype Enteritidis – Beckman Coulter 8000 platform. Accessed at: www.pulsenetinternational.org). Comparisons were performed using Bionumerics software version 5.01 (Applied Maths, Sint-Martens-Latem, Belgium) using the categorical coefficient and unweighted pair group method with arithmetic mean (UPGMA) clustering. The profiles were compared against the PulseNet USA national MLVA database.

Results

Isolation of Salmonella from different sources

Laboratory registers of stool specimens received in 2009 revealed 336 NTS cases of which 190 (56.5%) were *Salmonella* serogroup O:9. From 11,330 blood culture bottles received in 2009, 31 cases of proven NTS bacteraemia were identified of which 19 (61.3%) belonged to *Salmonella* serogroup O:9. Moreover, NTS were isolated from exudates from soft tissue and internal organs from 15 patients in 2009 and in 12 (80%) cases, the causative organisms was *Salmonella* serogroup O:9.

Records of the microbiological testing of 1,770 food specimens performed in 2009 showed 24 *Salmonella*-positive samples of which 16 (67%) were due to *Salmonella* serogroup O:9.

Results showing trends in the number of *Salmonella* serogroup O:9 from different sources are summarized in Figure 1.

Serotyping and Antimicrobial resistance

All isolates were identified as *S. Enteritidis* and were susceptible to ampicillin, apramycin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol,

gentamicin, nalidixic acid, neomycin, spectinomycin, sulphamethoxazole, tetracycline, and trimethoprim. One stool isolate (#8305) exhibited intermediate susceptibility to streptomycin.

Multiple-Locus Variable-number tandem repeat Analysis

Thirty seven isolates (78.7%) exhibited the MLVA allele profile 4-7-2-10-2-4-10. The remaining isolates exhibited four different profiles, all of which were highly related to the main profile, by differing at a single locus by 1 to 2 repeat units. All five profiles were rare in the United States MLVA database with prevalence ranging from 0.05%-0.16%. The main profile was found in 16 (89%) of 18 human stool isolates, six (67%) of nine blood culture isolates, three (75%) of four exudate isolates including an isolate recovered from a brain abscess, and 12 (75%) of 16 food isolates. Six (75%) of eight raw chicken isolates, including four (80%) of five from one major breeder, also had this profile. Moreover, all eight human and three food isolates linked to the large outbreak had this profile.

The second most common MLVA allele profile, 4-7-2-10-2-4-11, was exhibited by isolates recovered from mayonnaise samples taken eight months apart from a snack bar and the human isolate that was linked with food poisoning at this bar. Interestingly, this profile was also found in the only isolate from raw chicken marketed by another major breeder. An isolate from a parotid gland abscess also had this profile. The three other profiles were found mostly in isolates from blood cultures of patients with underlying diseases.

Discussion

A pandemic of *S. Enteritidis* was first noted in the late 1980's and has been attributed to infected poultry and contaminated eggs [13]. *Salmonella* serovar Enteritidis has been among the most prevalent serovars reported globally for several years. However, geographical variation in the isolation of *S. Enteritidis* has been noted. In 1995, *S. Enteritidis* was found to be the most frequently isolated serovar in about 88% of the countries in the European and American regions, but in none of the countries in the African region [14]. In 2000-2002, *S. Enteritidis* was again the most prevalent *Salmonella* serovar in humans globally and accounted for 85% of *Salmonella* cases in Europe, 38% in Asia and 26% in Africa [15], however, Mauritius was spared the pandemic until 2007. The reason for this is unclear but the fact that almost all chicken and eggs sold in Mauritius were locally

produced before 2008, is probably a significant factor. However, since 2008, *S. Enteritidis* has been commonly isolated from clinical and food samples and, indeed, in 2008 and 2009, non-typhoidal *Salmonella* serogroup O:9 displaced *Salmonella* serogroup O:4 as the most common serogroup recovered from human stools in Mauritius. There was also a simultaneous rise in the number of (non-typhoidal *Salmonella*) NTS isolates from blood cultures in 2008 and 2009, with *Salmonella* serogroup O:9 accounting for about 60% of the cases in these years. Isolation of any NTS from exudates had been infrequent in Mauritius until 2009 when almost three-quarters of NTS isolates from exudates were *S. Enteritidis*. Thus, following the emergence of *S. Enteritidis* in the country, more invasive infections have been encountered than previously, particularly in patients with underlying diseases. Previously published studies have shown that *S. Enteritidis* are among a few serotypes that are associated with a higher risk of extra-intestinal infection [16,17]. Immunocompromised patients are also known to be at higher risk for the development of bacteremia with this serotype [17]. However, some of our patients, particularly those with abscesses, were not immunocompromised and were not known to be suffering from underlying diseases.

The main objective of the present study was to investigate the genetic relatedness among *S. Enteritidis* isolates causing gastrointestinal illness, bacteremia and other extraintestinal infections in patients from Mauritius and to potentially identify the source of transmission to humans. Given the limited discriminatory power of pulse-field gel electrophoresis (PFGE) to differentiate strains of *Salmonella* serovar Enteritidis, a more discriminatory molecular subtyping method (MLVA) was utilized [18]. Retrospective data analysis identified a marked increase beginning in 2008, in both human NTS serogroup O:9 infections and the recovery of NTS serogroup O:9 from raw chicken and ready to eat food products in Mauritius. Although most isolates were not serotyped, the great majority were likely to have been *Salmonella* serovar Enteritidis as almost all of *Salmonella* serogroup O:9 isolates chosen for further studies were subsequently confirmed as this serotype.

Molecular and phenotypic subtyping of 47 representative isolates of *S. Enteritidis* revealed the presence of highly related strains in both humans and chicken, indicating that poultry is likely the main reservoir for human infections in Mauritius. Indeed, outbreaks caused by this serotype were associated

mostly with chicken or egg-based products as the vehicle although infected foodhandlers may have been involved in a few cases. In some cases cross-contamination with chicken or eggs in the kitchen may have led to contamination of other types of food such as chili sauce.

All eleven stool and food isolates linked with the large outbreak associated with chicken tikka or kebab were indistinguishable by MLVA typing. Similarly, with one exception, all isolates linked to an outbreak had indistinguishable profiles, suggesting that the same strain was present in both the food and the clinical samples. In the case of the outbreak associated with the snack bar, one raw chicken isolate had a slightly different MLVA allele profile from the stool and mayonnaise isolates, possibly because isolation of *S. Enteritidis* in that particular chicken specimen was an incidental finding unrelated to contamination of the mayonnaise which was the probable vehicle in the outbreak. Even though only slight differences were identified by MLVA among the 47 isolates, typing data generally supported epidemiological information available on isolates that were considered to be related.

The MLVA typing results suggest that all isolates are of a single clone which is most likely to have arisen from a single source. However, further analysis of the diversity in strains circulating in Mauritius would be necessary to confirm this. Although, *Salmonella* serovar Enteritidis is known for not being particularly resistant to antimicrobials, the lack of even a single resistance determinant also supports the hypothesis of a very clonal distribution. It is unclear how this clone was introduced in the country but according to some veterinarians, imported contaminated fishmeal for poultry is suspected as a possible source.

Stool isolates were highly related or indistinguishable from isolates recovered from blood and exudate. These results suggest that the same clone is responsible for both intestinal and extra-intestinal infections. The emergence and spread of this organism has caused considerable morbidity in the country. The financial impact is also significant as extra-intestinal infections typically result in many days of work lost and increased cost of hospitalization.

The presence of clonally related strains and the limited number of poultry breeders in Mauritius offer clear opportunities for epidemiologic interventions. Control of *Salmonella* in broilers from the affected poultry producer could lead to a marked reduction in the number of cases of salmonellosis in humans.

Control strategies could include the use of *Salmonella*-free parent flocks and *Salmonella*-free chicken feeds, regular cleaning and disinfection of poultry houses, with empty resting periods between flocks, measures to prevent domestic and wild animals, including wild birds, from gaining access to the premises, vaccination of breeder and layer flocks [19,20], and intensive flock-level testing with destruction of infected flocks [21]. However, apparently expensive measures could eventually be cost-effective as reduction of *Salmonella* in food animals would most likely lower the burden of human infections and reduce the cost associated with such illnesses.

Conclusions

Epidemiologic studies indicate that the burden of disease due to *S. Enteritidis* increased dramatically in 2008 and 2009. Characterization of clinical and food isolates revealed that a highly clonal strain of *S. Enteritidis* is circulating among the human population and it can also be found in food specimens originating from a major poultry breeder. The homology between stool and extra-intestinal isolates suggests that one strain is causing both gastrointestinal and invasive infections. Identification of a common strain circulating among the human population and poultry establishments offers a clear opportunity for intervention. Controlling *S. Enteritidis* in the poultry population can have a direct impact on public health in Mauritius.

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