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Prevalence, serotype and presence of invasion gene in *Salmonella* isolated from frog meat obtained from western region of Côte d'Ivoire

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ABSTRACT

This study was carried out to investigate the prevalence of *Salmonella*, serogrouping and presence of *invA* gene (*Salmonella* Invasion Gene A) of *Salmonella* strains obtained from *Hoplobatrachus occipitalis* in Côte d'Ivoire. A total of 210 fresh and 384 smoked frogs from three local market were evaluated for the presence of *Salmonella*. Biochemical identification, serotyping test and detection of *invA* gene by polymerase chain reaction (PCR) were performed. The results obtained indicate that all 384 smoked frog was not contaminated by *Salmonella* sp. while, 11.4% of fresh frog were positive for *Salmonella*. There was no significant difference between the contamination rate according to sites (P>0.05). The serotyping results obtained show that isolated strains belong to four serotypes and the most dominant serotype was *Salmonella lindenburg* (55.5%). PCR reaction showed that 86.7% of the *Salmonella* possessed *invA* gene. The presence of *Salmonella* in edible frog indicates that there is a health risk for consumers and this requires monitoring in order to minimize the risk of contamination to members of the public.

INTRODUCTION

Salmonella is the major cause of food borne infections, and the second most common food borne illness after *Campylobacter* infection (Meldrum and Wilson, 2007). It constitutes a major public health burden and cost of treatment is high in many countries. It has been estimated that *Salmonella* caused approximately one million cases of foodborne illness and 378 deaths per year in the United State (Scallan et al., 2011). *Salmonella* is one of the major foodborne pathogens worldwide (Soultose et al., 2003) and is responsible for outbreaks of foodborne illness in humans via cross-contamination and consumption of undercooked meats (Bailey and Cosby, 2003). Poultry products have been recognized to be the main source of this pathogen in humans (Karmi, 2013). Howerver, contact with toads or frogs clearly pose a potential risk for humans. Reptiles and amphibians are also a source of infection and may cause as many as 70,000 human cases annually in the United States (Mermin et al., 2004). The pathogens can survive in the meat until presented to the market (Redmond and Griffith, 2003).

In Côte d'Ivoire, frogs meat are traditionally used to supplement in the diet of different ethnic groups from the

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west where it serves as an important source of animal protein very highly appreciated by the consumers. Nowadays, frog meat consumption could be widespread to neighboring regions (west central and central) due to rural migration and ethno-cultural mixing of populations (Blé et al., 2016a).

Although many articles reported *Salmonella* strains in others animals like fish and chicken in Côte d'Ivoire (Adingra et al., 2010; Bonny et al., 2014). There is no information available regarding *Salmonella* from amphibians particularly frog (*Hoplobatrachus occipitalis*). Most studies on frogs focused on taxonomy, systematics, diet, and parasites (Tohé et al., 2014; Assemian et al., 2016). However, the study performed by Blé et al. (2016b) reported isolation of *Aeromonas* sp. rather than *Salmonella* from *H. occipitalis*. Based on the importance of *Salmonella* in human public heath, this study was undertaken to determine the prevalence, the serotype and presence of *InvA* gene in *Salmonella* isolated from frog in Midwest area of Côte d'Ivoire.

MATERIAL AND METHODS

Sampling

A total of 210 fresh frogs and 384 smoked frogs *H. occipitalis* were collected from in three markets located in Issia, Daloa and Sinfra (Midwest area of Côte d'Ivoire) during December 2015 to March 2016. Each frog was collected in sterile plastic bags in the morning between 7 and 9 and then transported to the laboratory in an ice chest for analysis. This specie was chosen because according to previous research, frogs mainly sold and consumed were *H. occipitalis* (Tohé et al., 2014).

On each fresh frog, three organs including intestine, skin and muscle were removed aseptically. For smoked frog, organ analyzed was muscle. All fresh specimen collected in the market appeared to be healthy as judged by skin color and luster.

Culture and identification of *Salmonella* strains

All strains were isolated according to the protocol described by standard ISO 6579 (ISO-6579, 2001). In the laboratory, 1 g of intestine and 5 g of muscle and skin from individual frog were cultured in 5 and 45 ml of peptone water buffer respectively, then incubated at 35±2°C for 24 h. After overnight incubation, 0.1 and 1 ml from pre-enrichment were respectively inoculated into 10 ml of Rappaport Vassiliadis broth (Bio-Rad, Marnes La Coquette, France) and 10 ml of Muller Kauffmann Tetrathionate broth. The tubes were incubated again at 42°C and 35±2°C for 24 h respectively for Rappaport Vassiliadis and Muller Kauffmann Tetrathionate broth. The contents of each tube were then streaked on surface of Hecktoen Enteric Agar (Himedia, Inde) and Xylose-Lysin-desoxycholate (XLD) agar (Himedia, India) and incubated at 35±2°C for 24 h. The presumptive *Salmonella* isolates wich are green colonies with black center in Hektoen Enteric Agar and red colonies with black center on XLD agar were confirmed using conventional biochemical tests like oxydase, catalase, citrate, glucose, lactose, H_2S, gaz, mannitol, mobility, lysine decarboylase, urea, indole and tryptophane desaminase of Le Minor and Richard (1993).

Salmonella isolates were further serotyped by agglutination method using antisera against O and H antigens (BioRad, Marnes-la-Coquette, France) and the Kauffmann-White classification pattern (Kauffmann, 1934; Le Minor and Popoff, 1997). The *Salmonella* strain was transplanted on nutrient agar and incubated at 35±2°C for 24 h. A strain was emulsified in physiological water. In case of agglutination, the strain is considered self-agglutinable and the protocol is stopped. Strain that was not self agglutinable was emulsified in antiserum so as to obtain homogeneous haze on a clean slide. The culture is taken on the slope of the agar for the detection of the O antigen or in the condensation water for the detection of the H antigen. The observation of agglutinates indicates a positive reaction.

Detection of 16S *rRNA* and *invA* gene

The extraction of the DNA was carried out according to the protocol of Smith et al. (2015). The 24 h strains were grown in 1 ml of brain heart broth (Bio-Rad, France) and incubated at 35±2°C for 24 h. After incubation, the tubes were centrifuged at 10,000 rpm for 5 min. A volume of 1 ml of milli-Q water was added to the pellet and centrifuged at 10,000 rpm for 5 min. The pellets were washed twice with sterile water successively under the same conditions. After this, 200 µl of milli Q water was added to the pellet and the tubes were mixed with the vortex (PV-1 Glove-bio) and boiled in a dry bath at 100°C for 10 min. The supernatant obtained after vortexing and centrifugation (12,000 rpm for 5 min) was stored at -20°C and used as a DNA template (Smith et al., 2015).

The reaction for all the PCR according to Smith et al. (2015) and Amini et al. (2010) protocol, was carried out in a 25 µl reaction mixture containing 10× PCR buffer (Promega, France), 1.5 mM Magnesium Chloride (Promega, USA), 1.25U Taq DNA polymerase (Promega, France), 200 µM of each dNTP (Sigma Aldrich), sterile water, 20 pMol and 1 µM of each primer respectively for 16S *rRNA* and *InvA*. Then, 2 µl of DNA was used in the PCR and the amplification was carried out in a Thermocycler (TECHNE).

The PCR conditions for 16S *rRNA* were: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 54.1°C and

Table 1. Sequence of primers used in this study.

Genes	Sequence of primers (5'-3')	size	References
16S rRNA	TGTTGTGGTTAATAACCGCACACAAATCCATCTCTGGA	574 bp	Smith et al. (2015)
*Inv*A	ACAGTGCTCGTTTACGACCTGAATAGACGACTGGTACTGATCTAT	244 bp	Chiu et al. (1996)

Table 2. Contamination rate of *Salmonella* in fresh frog according to different sites.

Sites	Frog analyzed	Frog contaminated	Rate of contamination (%)
Issia	84	6	7.1
Daloa	66	12	18.1
Sinfra	60	6	10.0
Total	210	24	11.4

1 min at 72°C and final extension for 10 min at 72°C. The *InvA* gene, the PCR conditions consisted of denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 2 min. The final extension was carried out at 72°C for 10 min. The amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and visualized in UV transilluminator (Cleaver Scientific LTD, Dominique dutscher, France). The primer sequences and theirs corresponding genes used in this study are given in Table 1.

Data analysis

All data were recorded and analyzed using the Statistical Package for Social Sciences (SPSS) Version 20.0 (IBM Corporation) software. Descriptive statistics such as prevalence, frequency and average were calculated for quantitative variables. For all measures of association, p-values <0.05 were considered significant.

RESULTS AND DISCUSSION

Characteristic *Salmonella* strains isolated on Hektoen and XLD agar were submitted to biochemical tests of Le Minor. The results obtained were: Oxydase (-); Catalase (+); Citrate (+); Glucose (+); Lactose (-); H_2S (+); Gaz (+); Mannitol (+); Mobility (+); LDC (+); Urea (-); Indole (-) and TDA (-), characteristic of *Salmonella* genus.

The results of this study revealed that there were no *Salmonella* (0 %) in the muscle of 384 smoked frogs analyzed. The absence of *Salmonella* in *H. occipitalis* in our study was consistent with findings of other study on *Lithobates catesbeianus* that reported a prevalence of 0% (Rodrigues et al., 2014). This absence indicates that frogs sold in the market were sufficiently smoked to minimize microbial growth. Also, this result could be due to the action of temperature and phenols contained in the smoke during curing of frog meat which may inhibit the formation of toxins and reduce the growth of bacteria as reported by Goueu (2006) and Adebayo-Tayo et al. (2015).

However, 24 (11.4%) of 210 fresh frogs were found to contains *Salmonella* strains (Table 2). These results are in agreement with the findings of Barreira et al. (2011) after study conducted on 30 frozen frog carcasses acquired in local market in Rio de Janeiro where level of contamination of *Salmonella* was 10.0%. Also, the values obtained in our study were lower than those obtained by Shin et al. (2012) who reported positive isolation in 18.4 and 28.6% of sample respectively by traditional detection and PCR method. The presence of *Salmonella* in samples analyzed indicates that sites of frog collection can be contaminated by a variety of microorganisms. This was predictable because frogs sold in markets are harvested in the insalubrious premises. Indeed, frog chain production is informal and all frogs are collected generally without distinction in swamps, in paddy fields, ponds and others irrigation system where frogs are known to presence.

The contaminate rate the organs analyzed was following: intestine (10.0%), skin (5.2%) and muscle (2.0%). This result confirms that *Salmonella* is naturally found in the gastrointestinal tract of amphibians such as frogs and these animals are healthy carriers potential of this bacterium as previously indicated by Gray (2011). The contamination of muscle observed could be attributed to cross-contamination during the technique of capture or handling of frog. Generally, frogs are harvested during night with the arrow. Sometime this practice tears up intestine, liver, heart and *Salmonella* in internal organs could probably contaminated the muscle and all carcass of frog.

Table 3. Prevalence and diversity of serotypes of *Salmonella* isolated from fresh frog.

Serotypes	Number of *Salmonella* isolate			Total (%)
	Muscle	Intestine	Skin	
S. ser Brezany (O:4)	0	2	4	6 (13.3)
S. ser Lindenburg (O:8)	3	16	6	25 (55.5)
S. ser Newport (O:8)	1	4	0	5 (11.1)
S. ser Enteritidis (O:9)	0	4	5	9 (20.0)
Total (%)	4 (8.9)	26 (57.8)	15 (33.3)	45 (100.0)

Table 4. Distribution of *InvA* in different serotypes of *Salmonella* isolated from fresh frog.

Serotypes of *Salmonella*	Presence of *InvA* gene N (%)
Salmonella ser Enteritidis	9 (100.0)
Salmonella ser Newport	5 (100.0)
Salmonella ser Lindenburg	21 (84.4)
Salmonella ser Brezany	4 (66.7)
Total	39 (86.7)

The highest percentage of contamination was found from Daloa site samples (18.1%) followedg by Sinfra (10.0%) and then Issia (7.1%) (Table 2). There was no significant difference (P>0.05) between the contamination rate according to sites. This result is in line with findings of Tracogna et al. (2013) who showed in their study that contamination of *Salmonella* did not also depend on the sampling site.

The serotyping revealed four different types of *Salmonella*: *Salmonella* ser. Lindenburg (55.5%) *S*. ser. Enteritidis (20%), *S.* ser. Brezany (13.3%) and *S*. ser. Newport (11.1%) (Table 3). The serotypage showed that the strains belonged to three serogroups of *Salmonella:* O:4 (B); O:8 (C) and O:9 (D) with prevalence of O:8 (53.6%), O:9 (16.1%) and O:4 (10.7%) respectively. Excepted *S*. Newport, all serotype were found on all sampling sites. The detection of different serovars in the same site of sampling suggests the heterogeneity of aquatic environment.

In this study, *S*. ser. Enteritidis strains (20.0%) were isolated. This disagrees with the work of Shin et al. (2012), who did not detect this serotype. Data on serotypes isolated in frogs are very old and limited. The previous work has shown a diversity of serotypes and serogroups of *Salmonella* isolated from frog organs. Andrews et al. (1977) detected 30 serovars of *Salmonella* and Rajagopalan et al. (1985) demonstrated 14 serotypes in isolated frog legs. The most serotypes identified in their study were: *S*. ser. Newport (C2), *S*. ser. Enteritidis (D), *S*. ser. Arizona, *S*. ser. Bareilly (C1), *S* ser. Abony, *S*. ser. Hvittingboss (I), *S*. ser Poona (G), *S*. ser. Mgulani (P), *S*. ser. Typhimurium (B) and *S*. ser. Anatum (E).

The data obtained in this study are very important and significant to human health in areas where frog meat is consumed. Indeed, *Salmonella* from group D and B account for approximately two-thirds of all reported *Salmonella* infections (Mermin et al., 2004). *S*. *enteritidis* is highly pathogenic and most often involved in severe cases of poisoning in the world (Herikstad et al., 2002). In addition, an outbreak of *S. enteritidis* and *typhimurium* declared in more than 30 US states was due to contacts with the African dwarf frog (CDC, 2010). In United States from 2002 to 2004, several outbreaks of *S. newport* after contacts with the sheep have also been reported (Lazarus et al., 2007). Recent data reported 90 000 cases of salmonellosis per year in the United States linked to contact with amphibians and reptiles (Gray, 2011). For all these reasons, children, elderly or people with weakened immune systems have been forbidden to touch or handle the frog to avoid the risk of contamination with *Salmonella* (CDC, 2010). Note that all serovars are potentially pathogenic to humans and particularly responsible for food-borne infections or healthy carrier (Elgroud, 2009).

All strains biochemically identified like Salmonella were confirmed by PCR with amplification of 574 bp fragments of 16S *rRNA* gene specific for *Salmonella* (Figure 1). The detection of virulence genes showed also the presence of invasive *InvA* gene (244 bp) in 86.7% of *Salmonella* strains (Figure 2). This gene was found in all strains of *S. enteritidis* and *S. newport* (Table 4). This finding is at

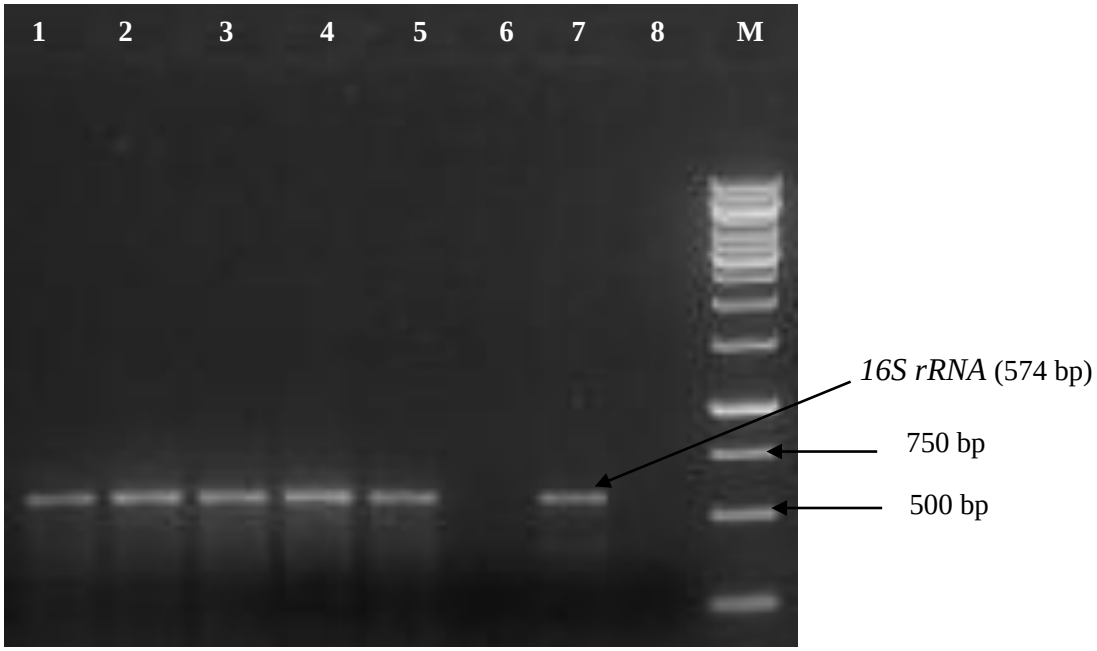

Figure 1. Electrophoretic profile of amplification product of *16S rRNA* gene of *Salmonella* isolated from fresh *H. occipitalis.*
M: DNA size marker (1 kpb DNA Ladder, Ogenruler); **Lane 1-2:** *S*. ser Lindenburg; **Line 3:** *S*. ser Brezany; **Lane 4:** *S*. ser Newport; **Lane 5:** *S*. ser Enteritidis; **Lane 7:** *Salmonella* ATCC14028; Lane 6-8: Negative control.

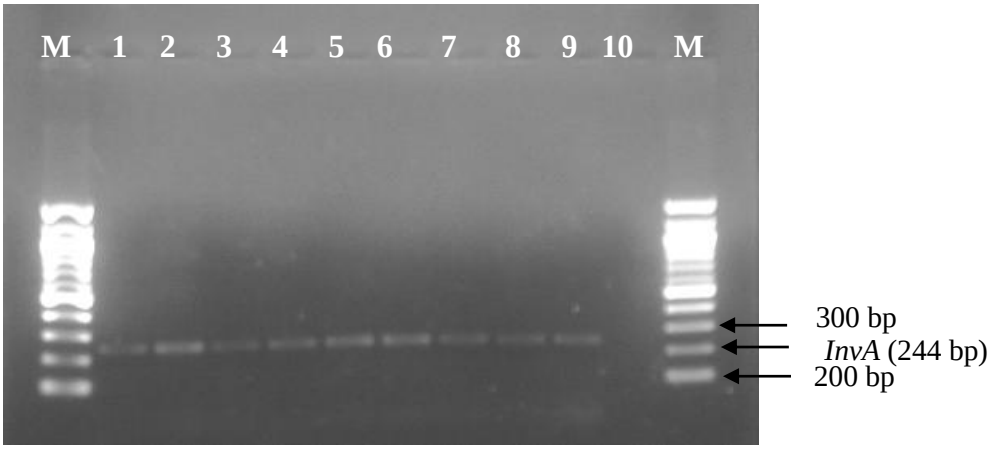

Figure 2. Electrophoretic profile of amplification product of *InvA* gene of *Salmonella* isolated from fresh *H. occipitalis.*
M: DNA size marker (100 bp DNA Ladder, Invitrogen); **Lane 1-2:** *S*. ser Lindenburg; **Lane 3-4:** *S*. ser Brezany; **Lane 5-6:** *S*. ser Newport; **Lane 7-8:** *S*. ser Enteritidis; **Lane 9:** *Salmonella* ATCC14028; **Lane 10:** Negative control.

variance with the reports of Amini et al. (2010) and Koochakzadeh et al. (2015) who detected the *InvA* gene in all *Salmonella* isolates that they tested. The *InvA* gene is involved in the invasion of intestinal epithelial cells and is responsible for the virulence of *Salmonella* (Zou et al., 2012). The detection of the gene in the *Salmonella* isolated implies the organisms are virulent and will be able to penetrate host epithelial cells, causing infection. Consequently, this high prevalence of *InvA* gene in frog samples represents a health risk for frog consumer. This

risk may further be higher if frog meat are consumed undercooked or cross contamination in the kitchen with *Salmonella* during meal preparation. Immunocompromised individuals, the elderly and young children are the most susceptible population to be exposed to possible cases of salmonellosis (Ruiz and Rodriguez, 2003).

Conclusion

Fresh frog meats were contaminated with pathogenic *Salmonella* (11.4%) and four serotypes were found. The *Salmonella* isolates (86.7%) were positive for the presence of invasion gene *Inv*A. This study reveals that frog meat may represent health risk for consumers. Consequently, it is necessary to educate the public on hygienic measures during harvesting, handling and consumption of frog which are possible infection sources of *Salmonella*. Monitoring is required in order to minimize the risk of contamination to exposed persons.

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