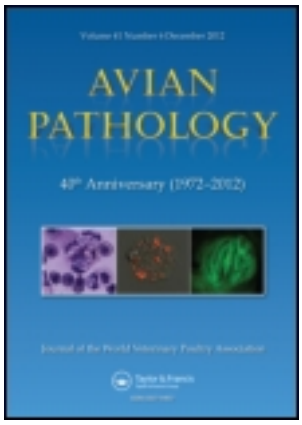

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Identification and typing of *Salmonella enterica* serotypes isolated from guinea fowl (*Numida meleagris*) farms in Benin during four laying seasons (2007 to 2010)

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The main problem for the local guinea fowl (*Numida meleagris*) traditional farming and raising system in north-east Benin is the high mortality rate of the keets (up to 70%) due to a combination of climatic, nutritional, hygienic and infectious causes. The present study was carried out to identify and compare the isolates of *Salmonella enterica* from necropsied keets, laying guinea fowl, surrogate hen mothers, other contact animal species and farmers during four laying seasons (2007 to 2010). *S. enterica* belonging to eight different serotypes (Adelaide, Farakan, Kingston, Legon, Luke, Oakland, Sangalkam and Teshie) and one untypable isolate were isolated from 13 to 19% of the necropsied keets. The serotypes Adelaide, Farakan, Luke, Sangalkam and Teshie and the untypable isolate were isolated in only one township during 1 year of sampling, while serotypes Oakland, Legon and Kingston were present in two to three townships for 2 to 3 years of sampling. Serotypes Farakan, Kingston, Legon, Oakland and Sangalkam were also isolated from faecal samples of laying guinea fowl and/or surrogate domestic fowl hen mothers. Further comparison by pulsed-field gel electrophoresis and virulotyping provided evidence for their clonality within each of those five serotypes and therefore for the adult guinea fowl and/or hens as the most probable origin of contamination of the keets. The antibiotic resistance profiles, with all isolates resistant to oxacillin, sulfamethoxazol and colistin, emphasize the rise of antibiotic resistance in salmonellas from guinea fowl in this area and the need for alternative therapy policies for these birds.

Introduction

To increase the daily food ration in developing countries it is imperative to identify the best means to stimulate local agricultural and animal production. Development of poultry production is one of the solutions for increasing animal protein production, especially in sub-Saharan Africa (Boko *et al*., 2012).

As part of current animal policy development, the Benin government is encouraging local guinea fowl production (*Numida meleagris*), particularly in the Borgou department in north-east Benin, where the raising of this poultry species is of high socio-economic importance for the population. guinea fowl are raised according to the scavenging system, using local surrogate domestic fowl (*Gallus gallus*) hens for incubation and hatching of the guinea fowl eggs and for raising the keets until weaning age (2 months). In this way, the female guinea fowl can continue to lay eggs without any interruption. The main problem with this traditional raising system is the high mortality rate of the keets, up to 70%, with clinical signs such as loss of appetite, prostration, chills, somnolence, whitish diarrhoea, dehydration, lameness and respiratory distress (Boko, 2004; Boko *et al*., 2011a, 2012).

Several bacterial species have been isolated from dead keets (Bessin *et al*., 1998; Boko, 2004; Boko *et al*., 2011a) but they mostly represent members of the resident commensal microbiota, which invade the body around or after death, or else they are secondary pathogens benefiting from favourable conditions (stress, weakening, cold, viral or mycoplasmal infection, heavy parasitism). One exception is the *Salmonella enterica* species, of which some serotypes are recognized avian

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primary bacterial pathogens, like Gallinarum/Pullorum in poultry (Euzeby, 1997; Bornert, 2000; Villate, 2001; Shivaprasad & Barrow, 2008).

Salmonellas are moreover important zoonotic pathogens, which can cause different serious diseases, such as enteritis, septicaemia, abortions, and so forth, in animals and humans, but also asymptomatic infections, or a healthy carrier state, mostly in the intestinal tract, liver, gallbladder and/or mesenteric lymph nodes (Bäumler *et al.*, 1998; Coburn *et al.*, 2007; Andrews-Polymenis *et al.*, 2010). Excretion of salmonellas causes widespread contamination both of the environment and of foods and feeds (Oosterom, 1991).

The purpose of this study was to identify and compare *Salmonella* isolates from different field samples collected in guinea fowl breeding farms in Benin during four laying seasons (2007 to 2010), by biotyping, serotyping, pulsed field gel electrophoresis analysis, virulotyping, and antibiotyping.

Materials and Methods

Sampling and isolation of *Salmonella*. Thirty-nine guinea fowl breeding farms in six townships (Banikoara, Bembèrèkè, Kandi, N'Dali, Parakou, Tchaourou) of the Borgou department were included in the survey (Figure 1). The field samples were collected from 75 guinea fowl eggs with dead embryos, from 199 2 month-old dead keets at necropsy and from faecal samples from 171 guinea fowl, 171 surrogate hens, 55 farmers and caretakers, 36 mice, and six ducks.

The isolation and identification procedure used for salmonellas was based on the International Organization for Standardisation norm 6579:2002 (ISO, 2002). Faecal samples (1 g) were initially pre-enriched by overnight incubation in 9 ml buffered peptone water at 37°C. For selective enrichment, 0.1 ml buffered peptone water broth or 1 g dead embryos or intestinal and caecal contents, liver and/or lungs of necropsied keets, were inoculated into 10 ml Rappaport–Vassiliadis broth and incubated at 42°C for 24 h. A loop full of the Rappaport–Vassiliadis-enriched culture was then plated onto two xylose–lysine–desoxicholate plates (Oxoid, Basingstoke, UK) and two *Salmonella–Shigella* agar plates and incubated at 37°C for 24 h. At the same time, one drop of aseptically collected heart blood at necropsy was directly streaked onto two 5% sheep blood Columbia and two MacConkey's agar plates (Oxoid) and incubated at 37°C for 24 h. Three suspected *Salmonella* colonies per plate were transferred onto nutrient agar plates and subsequently subjected to pre-identification tests: Gram staining, catalase and peroxidase production, the oxidation/fermentation test, production of indol and H_2S, and fermentation of glucose, lactose and urea (Barrow & Feltham, 1993; Quinn *et al.*, 2011). All agar plates and broth media were prepared from dehydrated powders (Oxoid) at the Bacteriological Department of the Veterinary Diagnostic and Serological Monitoring Laboratory of Parakou (Benin) where pre-identification tests were also performed.

Biotyping and serotyping. One pre-identified colony per sample was further tested for confirmation with the API20E® kit (Biomérieux, Marcy l'Etoile, France) and with OM immune sera (OMA, OMB, OMC; Bio-Rad, Nazareth Eke, Belgium) at Université de Liège (import permit numbers 145749, 380852, 569716). Confirmed *S. enterica* isolates were referred to CODA-CERVA and to ISP-WIV for full serotyping. Serotyping was carried out by slide agglutination with commercial mono-specific antisera (Bio-Rad) following the Kauffmann–White scheme (Grimont & Weill, 2007).

Pulsed field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) analysis was performed according to the Pulse Net Europe protocol (www.cdc.gov/pulsenet) (Bertrand *et al.*, 2010). When identical *Salmonella* serotypes were present in dead keets and in any other sample, the genomic DNA was extracted and digested with restriction enzyme *Xba*I (Fermentas, St Leon Rot, Germany). If the serotypes were present in the ISP-WIV reference collection, one strain of each serotype was added as a control strain and *Xba*I-digested *Salmonella* Braenderup H9812 strain DNA was used as a size marker. The Fingerprint II Informatix Software (Bio-Rad, Hercules, California, USA) was used to compare the PFGE profiles. The bands generated were analysed by using the Dice coefficient and the unweighted pair group method with averages with a tolerance of 1%.

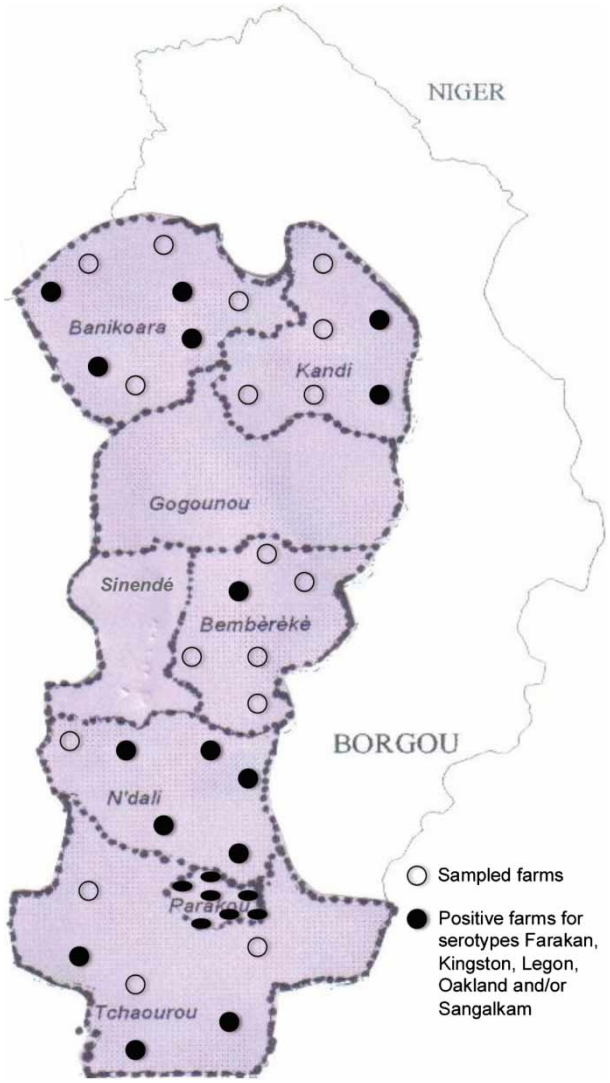

Figure 1. *Localization of the 39 sampled farms in the six townships of the Borgou department and of the 22 farms where* S. enterica *serotypes Farakan, Kingston, Legon, Oakland and Sangalkam were isolated.*

Polymerase chain reaction virulotyping. All confirmed *Salmonella* isolates were screened by polymerase chain reaction (PCR) for the presence of several virulence-associated genes (Table 1) located on *Salmonella* Pathogenicity Islands (SPI) 1 to 5 (Cortez *et al.*, 2006; Courtney *et al.*, 2006; Gassama-Sow *et al.*, 2006; Skyberg *et al.*, 2006; Hughes *et al.*, 2008): *prgH*, *invA*, *sitC*, *spaI*, *invE* (SPI-I); *spiC*, *ssaU*, *ttrB* (SPI-2); *mgcT*, *misL* (SPI-3); *orfL* (SPI-4); *pipD* (SPI-5). The DNA content was extracted by the boiling procedure (China *et al.*, 1996) and DNA amplification was carried out in a Mastercycler (Eppendorf, Le Pecq, France). The primer sequences and the amplification parameters have already been published (Cortez *et al.*, 2006; Courtney *et al.*, 2006; Hughes *et al.*, 2008), except those for the *invE*, *ssaU* and *mgcT* genes (Table 1). Four *Salmonella* Typhimurium strains were used as the controls (kindly provided by Prof. Georges Daube, Food Science Department, Veterinary Faculty, University of Liège).

The amplified DNA fragments were visualized after electrophoresis in a 1.5% agarose gel in Tris-acetate–ethylenediamine tetraacetic acid (TAE) buffer under ultraviolet light at 365 nm (Proxima 16 Phi + ;

Table 1. *Primers and amplification parameters of the PCR developed during this study for the virulotyping of the* Salmonella *isolates.*

SPI	Target gene	Primer sequence (5′→3′)	Denaturation temperature/time	Annealing temperature/time	Extension temperature/time	Number of cycles
SPI-1	*invE*	F: ACCGAAGGCGAAGCAAATAT R: GCTGTTCTGCCATTTCATGC	94°C/60 sec	57.9°C/60 sec	72°C/60 sec	40
SPI-2	*ssaU*	F: TGCTTTATTAGAGTCACTGA R: TTATGGTGTTTCGGTAGAAT	94°C/60 sec	64.6°C/60 sec	72°C/60 sec	30
SPI-3	*mgcT*	F: GTGTTATGCGGCTTAGGGCA R: CTCCAGTGAATTGCGGTGAT	94°C/60 sec	62°C/60 sec	72°C/60 sec	30

ISOGEN Live Science, De Meen, the Netherlands). The molecular weight marker was the SmartLadder MW-1700–10 (Eurogentec, Seraing, Belgium), ranging from 200 base pairs to 10 kilobase pairs.

Antibiotic sensitivity assay. The *in vitro* sensitivity patterns of all confirmed *S. enterica* isolates to 12 antimicrobial agents were determined by the disc diffusion method on Mueller–Hinton agar plates (Becton Dickinson, Erembodegem, Belgium) (Bauer *et al*., 1966). The antimicrobial discs contained antibiotics that are frequently used in poultry flocks in Benin: ampicillin (10 µg), oxacillin (5 µg), cefuroxim (30 µg), neomycin (30 µg), gentamicin (10 µg), colistin (10 µg), tetracyclin (30 µg), sulfamethoxazol (250 µg), trimethoprim/sulfamethoxazol (1.25 µg/23.75 µg), nalidixic acid (30 µg), enrofloxacin (5 µg) (Becton Dickinson), and flumequin (30 µg) (Oxoid, Dardilly, France). The inhibition zones were interpreted using the Antibiogramme Committee of the French Society for Microbiology (CASFM) guidelines for susceptibility testing (Soussy, 2010).

Results

Bacteriology. A total of 66 isolates were confirmed as being *S. enterica*: 31 from necropsied keets, three from dead embryos, 11 from laying guinea fowl faeces, 18 from surrogate hen mother faeces, two from farmer faecal samples and one from a mouse faecal sample (Table 2). Sixty-five of them could be serotyped and they were found to belong to 15 different serotypes (Table 3). Five serotypes (Farakan, Kingston, Legon, Oakland and Sangalkam) were isolated from necropsied keets, dead embryos, and faecal samples from guinea fowl and/or hens, in 22 farms out of the 39 sampled (Figure 1). The serotypes Farakan and Sangalkam were isolated in only one township during 1 year of sampling, while serotypes Oakland, Legon, and Kingston were present in two to three townships for 2 to 3 years of sampling (Table 3). The serotypes Adelaide, Luke and Teshie and the untypable isolate were only isolated from dead keets and/or embryos, while the serotypes Agama, Typhimurium var. Copenhagen, Uganda and Nima were only isolated from faecal samples of guinea fowl and/or hens (Table 3). *S. enterica* isolates of serotypes Kingston and Legon belonged to two different biotypes, and *S. enterica* isolates of serotype Oakland to three different biotypes (Table 3), while isolates from the other serotypes belonged to only one biotype.

Pulsed field gel electrophoresis. The *Xba*I PFGE profiles of the 51 *S. enterica* isolates of Farakan, Kingston, Legon, Oakland and Sangalkam serotypes isolated from necropsied keets, dead embryos, and faecal samples from guinea fowl and/or hens (Table 3) were different between the serotypes (Figure 2a to e). A very high level of similarity was recorded between isolates within serotypes Farakan (98%), Kingston (99%), Legon (98%), and Sangalkam (100%), respectively. On the other hand, two PFGE similarity groups were recorded within the serotype Oakland (Figure 2d): either profile was present in different townships, but profile 1 was isolated only in 2008 (14 isolates), while profile 2 was observed in 2007 (four isolates), 2008 (one isolate) and 2010 (eight isolates). The profile 1 Oakland isolates belonged to one biotype and the profile 2 isolates to the other two biotypes (Table 3). The PFGE profiles of the *Salmonella* Kingston and Legon control strains were different from the profiles of the isolates from Benin (Figure 2b, c), while the profile of the *Salmonella* Oakland control strain was identical to profile 1 of the isolates from Benin (Figure 2d). No *Salmonella* Farakan or Sangalkam control strains could be tested.

Virulotyping. All isolates belonging to the five serotypes tested positive by PCR for most of the target genes. One Farakan, one Kingston, 12 Oakland and one Sangalkam isolates tested negative with the PCR for the *ssaU* gene. Moreover the PCR for the *sitC* and *spaI* genes were also negative with one of the *ssaU*-negative Oakland isolates. No relationship was observed between the virulotyping results of the two PFGE profiles of the Oakland serotype isolates.

Antibiotyping. All isolates were resistant to oxacillin, sulfamethoxazol and colistin, while they all were

Table 2. *Prevalence of* S. enterica *isolates in field samples from 2007 to 2010.*

Type of sample	Year of sampling	Number of field samples	Number of positive samples (%)
Dead keet tissues	2008	29	14 (13.8)
	2009	42	8 (19.0)
	2010	38	5 (13.1)
	Total	202	31 (15.3)
Guinea fowl eggs	2007	75	3 (4.0)
	Total	75	3 (4.0)
Laying guinea fowl faeces	2008	64	5 (7.8)
	2009	60	4 (6.6)
	2010	47	2 (4.2)
	Total	171	11 (6.4)
Surrogate hen mother faeces	2008	64	9 (14.0)
	2009	60	6 (10.0)
	2010	47	3 (6.4)
	Total	171	18 (10.5)
Human faeces	2009	55	2 (3.6)
Mouse faeces	2009	36	1 (2.8)
Duck faeces	2009	4	0 (0)
	2010	2	0 (0)
	Total	97	3 (3.1)

Table 3. *Serotypes and biotypes of* S. enterica *isolated from field samples in six townships of Borgou department from 2007 to 2010.*

S. enterica serotype	Serological group	Biotype	Field samples	Geographical origin	Number of isolates (year)
S. Adelaide	O	670475257	Dead keet caecum	Parakou	1 (2009)
S. Agama	B	670455257	Laying guinea fowl faecal sample	N'Dali	1 (2009)
			Surrogate hen mother faecal sample	N'Dali	1 (2009)
S. Farakan	M	670475217	Dead keet caecum	Banikoara	4 (2008)
			Surrogate hen mother faecal sample	Banikoara	3 (2008)
S. Kingston	B	470455217	Dead keet caecum	Bembéréké, Tchaourou	2 (2008)
			Dead keet liver	Parakou	1 (2008)
			Surrogate hen mother faecal sample	Parakou	1 (2008)
		670455257	Dead keet caecum	Parakou	2 (2009)
S. Korlebu	E4	670455257	Farmer/caretaker faecal sample	Bembéréké	2 (2009)
S. Legon	B	670475257	Guinea fowl egg	N'Dali	2 (2007)
			Dead keet liver	Parakou	1 (2007)
			Dead keet caecum	N'Dali	2 (2009)
		470475217	Surrogate hen mother faecal sample	Parakou	1 (2008)
S. Luke	X	670455257	Dead keet liver	N'Dali	2 (2009)
S. Nima	M	670455257	Surrogate hen mother faecal sample	Banikoara	2 (2009)
S. Oakland[a]	C1	670451217[b]	Dead keet caecum	Banikoara, N'Dali, Parakou	4 (2008)
			Dead keet liver	N'Dali, Parakou	2 (2008)
			Laying guinea fowl faecal sample	N'Dali, Parakou, Tchaourou	5 (2008)
			Surrogate hen mother faecal sample	Parakou	3 (2008)
		670451257[c]	Guinea fowl egg	Parakou	1 (2007)
			Dead keet caecum	N'Dali	1 (2007)
			Dead keet liver	Parakou	1 (2007)
			Dead keet caecum	Parakou, Tchaourou	4 (2010)
			Laying guinea fowl faecal sample	Parakou	2 (2010)
			Surrogate hen mother faecal sample	Parakou	1 (2008)
			Surrogate hen mother faecal sample	Tchaourou	2 (2010)
		670475257[c]	Dead keet caecum	Parakou	1 (2007)
S. Saint Paul	B	670475257	Surrogate hen mother faecal sample	Bembéréké	1 (2009)
S. Sangalkam	D2	670455257	Dead keet caecum	Kandi	1 (2009)
			Laying guinea fowl faecal sample	Kandi	3 (2009)
			Surrogate hen mother faecal sample	Kandi	1 (2009)
S. Stanley	B	670455257	Mouse faecal sample	Bembéréké	1 (2009)
S. Teshie	X	670455317	Dead keet caecum	Kandi	1 (2008)
S. Typhimurium var. Copenhagen	B	670455257	Surrogate hen mother faecal sample	Parakou	1 (2009)
S. Uganda	E1	670455257	Surrogate hen mother faecal sample	Tchaourou	1 (2010)
S. UT[d]	D2	670455257	Dead keet caecum	Banikoara	1 (2010)

[a]Biotypes were identified using the API20E® kit (Biomérieux, Marcy l'Etoile, France) according to the following 27 tests: production of β-galactosidase, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase (ONPG, ADH, LDC, ODC), growth with citrate as carbon source (CIT), production of H_2S, urease, tryptophane deaminase and indol (H_2S, URE, TDA, IND), Voges–Proskauer test (VP), gelatine hydrolysis (GEL), acidification from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdaline and arabinose (GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA), production of peroxidase, nitrite and nitrogen (OX, NO_2, N_2), motility (MOB), growth on McConkey's agar (McC) and oxidation/fermentation test (OF/O, OF/F). The results are combined by three with each member of one triplet being given a value of 1, 2 or 4, respectively, when the result is positive. The total score of each triplet varies between 0 and 7 (Willcox *et al.*, 1980). [b]*Xba*I PFGE profile 1. [c]*Xba*I PFGE profile 2. [d]Untypable isolate.

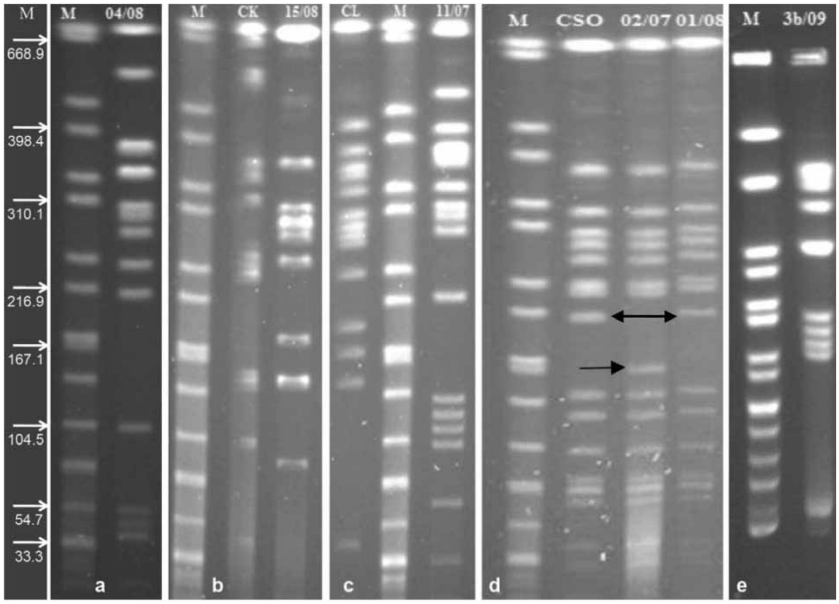

Figure 2. Xba*I PFGE profiles of the* Salmonella *isolates belonging to the five serotypes isolated from dead keets, keet embryos, laying guinea fowl faecal samples and/or surrogate hen mother faecal samples. 2a:* Salmonella *Farakan: field isolate 04/08. 2b:* Salmonella *Kingston: CK = control strain (ISP-WIV collection); field isolate 15/08. 2c:* Salmonella *Legon: CL = control strain (ISP-WIV collection); field isolate 11/07. 2d:* Salmonella *Oakland: CSO = control strain (ISP-WIV collection; profile 1); field isolate 02/07 (profile 2); field isolate 01/08 (profile 1). Black arrows point out the difference between profile 1 and profile 2. 2e:* Salmonella *Sangalkam: field isolate 3b/09. M =* Salmonella *Braenderup weight marker approximate sizes after* Xba*I digestion): 1135 kilobase pairs (kbp), 668.9 kbp, 452.7 kbp, 398.4 kbp, 336.5 kbp, 310.1 kbp, 244.4 kbp, 216.9 kbp, 173.4 kbp, 167.1 kbp, 138.9 kbp, 104.5 kbp, 78.2 kbp, 54.7 kbp, 33.3 kbp, 28.8 kbp, 20.5 kbp (Hunter* et al.*, 2005; Bertrand* et al.*, 2010).*

susceptible to trimethoprim/sulfamethoxazol (Figure 3). Thirty-six isolates expressed resistance to one additional antibiotic—cefuroxim (30 isolates), ampicillin (four isolates), or flumequin (two isolates)—and six isolates to two additional antibiotics—cefuroxim and either ampicillin (five isolates) or nalidixic acid (one isolate). No relationship was observed between the serotypes and the resistance profiles.

Discussion

S. enterica is considered an important pathogen in animals although different non-infectious causes related to climate, hygiene, overcrowding, underfeeding, insufficient water, and traumatic lesions, as well as other infectious causes (parasites, mycoplasmas and viruses), synergistically participate in the development of disease (Gast, 2008; Boko *et al.*, 2011a). During this 4-year survey (2007 to 2010), the yearly rate of isolation and identification of *Salmonella* from necropsied keets in the six townships of the Borgou department in north-east Benin was found to be between 13 and 19% (Table 2), in agreement with earlier results (Boko *et al.*, 2011a). Surprisingly, none of the 34 isolates from dead keets or embryos belonged to recognized avian primary pathogenic serotypes, such as Gallinarum/Pullorum,

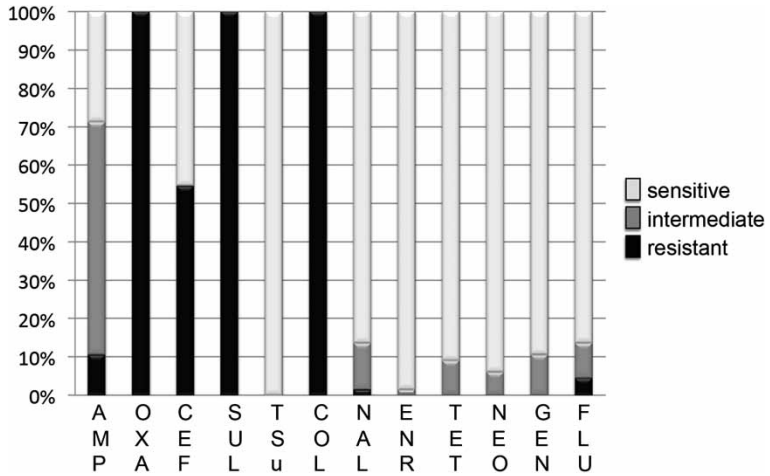

Figure 3. *Prevalence of antibiotic resistance amongst the 66* S. enterica *isolates. AMP = ampicillin; OXA = oxacillin; CEF = cefuroxim; SUL = sulfamethoxazol; TSu = trimethoprim/sulfamethoxazol; COL = colistin; NAL = nalidixic acid; ENR = enrofloxacin; TET = tetracyclin; NEO = neomycin; GEN = gentamicin; FLU = flumequin.*

Typhimurium or Typhimurium var. Copenhagen, (Euzeby, 1997; Bornert, 2000; Villate, 2001; Shivaprasad & Barrow, 2008), but to eight "exotic" serotypes (Adelaide, Farakan, Kingston, Legon, Luke, Oakland, Sangalkam, Teshie), and one isolate could not be serotyped. It is therefore important to confirm that these serotypes are actual pathogens in guinea fowl, to identify the source of contamination, and to speculate about the zoonotic potential of the different serotypes.

The pathogenicity of the serotypes Farakan, Kingston, Legon, Oakland, and Teshie was assessed experimentally by oral challenge of 2-week-old keets (Boko *et al.*, 2011b). These five *Salmonella* serotypes are not only able to colonize keet intestines, but also to cause general clinical signs similar to, although less dramatic than, those observed during field surveys. This emphasizes the contribution of other infectious agents and of non-infectious causes to the high morbidity and mortality scores observed in field conditions. The intensity of the clinical signs was also significantly different between the serotypes, suggesting that they may differ in terms of other properties related to their relative adaptation to 2-week-old guinea fowl and/or by presence/absence of some SPI-located virulence-related genes. Nevertheless, the virulotyping results do not reveal any clear-cut difference between the serotypes, and indeed confirm that the great majority of the *S. enterica* isolates that were tested possess the genes necessary not only for enteropathogenicity but also for the invasion of the bloodstream and internal organs (Darwin *et al.*, 1999; Marcus *et al.*, 2000; Chakravortty *et al.*, 2002; Hensel, 2004; Schmidt & Hensel, 2004; Cornelis, 2006; Barrow *et al.*, 2010; Akopyan *et al.*, 2011; Malik-Kale *et al.*, 2011).

The comparison by biotyping, pulsed field gel electrophoresis analysis and virulotyping of the *S. enterica* serotypes Farakan, Kingston, Legon, Oakland, and Sangalkam isolates from necropsied keets, dead embryos and faeces from laying guinea fowl and/or surrogate hen mothers highlights their high degree of homogeneity. The only variation was observed within the serotype Oakland, with two quite closely related pulsotypes (Figure 2d; Tenover *et al.*, 1995) found to be to some extent related to the three biotypes and to the year of isolation, but not to the virulotypes or to the host (Table 3). Therefore, the origin of the infection of young keets by those five serotypes is most likely to reside in the intestinal contamination and faecal excretion of adult guinea fowl and/or hens following a faecal–egg or a faecal–oral route of contamination. Such horizontal transmission of salmonellas between adult poultry and keets is of course favoured by several local traditions (Belco, 1985; Bessin *et al.*, 1998; Dahouda, 2003; Boko *et al.*, 2011a, 2012): the raising of guinea fowl according to the scavenging system, with the keets walking freely around the coops and following the surrogate hen mothers until weaning age (2 months) along with the chicks; the offering of guinea fowl as presents to honour socially important guests, such as in-laws or strangers; the purchase in local markets of poultry coming from other townships or departments and their immediate placing without any quarantine in the backyard in close contact with other poultry; and so forth. Such social and commercial practices may also explain the differences in isolation yearly rates of the different serotypes and the resurgence of some serotypes in different townships for 2 to 3 years of sampling (Table 3).

Another very important concern is the zoonotic potential of these "exotic" *S. enterica* serotypes. They were reported for the first time in humans or in foodstuffs and animal products in equatorial and tropical countries (Caribbean Islands and Africa; Le Minor *et al.*, 1975), but have since been identified very rarely in Europe and North America in either humans ($<0.01\%$) or animals (Ball *et al.*, 1964; Boqvist *et al.*, 2003; CDC, 2009; HPA, 2012). Many more isolates of those serotypes should of course be compared, but at this stage we cannot completely rule out that guinea fowl might represent a source of *S. enterica* infection for humans. Indeed, although the *Salmonella* Kingston and Legon control strains have a different PFGE profile compared with the keet isolates (Figure 2b,c), the control strain of *Salmonella* Oakland belongs to the PFGE profile 1 of the guinea fowl isolates (Figure 2d). Unfortunately information on the actual origin of the control strains from the ISP-WIV collection is no longer available.

The antimicrobial susceptibility patterns identified here demonstrate the increase in antibiotic resistance, with only the association trimethoprim/sulfamethoxazol still active on all isolates. The reduction in susceptibility to flumequin in particular must be regarded as an alarm signal, since this fluoroquinolone is the last-resort antibiotic used by farmers to treat any disease problems in guinea fowl without any laboratory diagnosis. The only alternative is phytotherapy using local plant extracts (Nakamura *et al.*, 1999; Adebolu & Oladimeji, 2005; Adiguzel *et al.*, 2005; Bassolé *et al.*, 2010). This approach relies on local population tradition but its actual value in treating bacterial disease has not yet been proved.

In conclusion, this study in Benin highlights the role of several local traditions in the raising of guinea fowl, such as the scavenging system, the purchase at local markets and the absence of quarantine in favouring *S. enterica* infections of young keets originating from the faeces of laying guinea fowl and surrogate hen mothers that also play a role in the persistence of *S. enterica* in the farms. More transverse and longitudinal surveys in sub-Saharan African and other developing countries are necessary to compare and to follow the evolution of the different serotypes present in guinea fowl, especially in keets, and to detect the emergence of still unidentified ones. Similar studies in developed countries should also be performed to confirm, or not, specific geographic and/or host association(s) of the identified serotypes in Benin.

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References

Adebolu, T.T. & Oladimeji, S.A. (2005). Antimicrobial activity of leaf extracts of *Ocimum gratissium* on selected diarrhoea causing bacteria in southwestern Nigeria. *African Journal of Biotechnology*, 4, 682–684.

Adiguzel, A., Gulluce, M., Sengul, M., Ogutcu, H., Sahin, F. & Karaman, I. (2005). Antimicrobial effects of *Ocimum basilicum* (Labiatae) extract. *Turkish Journal of Biology*, 29, 155–160.

Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H. & Fallman, M. (2011). Translocation of surface-localized effectors in type III secretion. *Proceedings of the National Academy of Sciences USA*, 108, 1639–1644.

Andrews-Polymenis, H.L., Baumler, A.J., McCormick, B.A. & Fang, F.C. (2010). Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. *Infection and Immunity*, 78, 2356–2369.

Ball, M.M., McWhorter, A.C. & McConnaughey, J. (1964). An undescribed *Salmonella* serotype: *S.* Oakland. *International Bulletin of Bacteriological Nomenclature and Taxonomy*, 14, 65–66.

Barrow, G.I. & Feltham, R.K.A. (Eds) (1993). Characters of Gram-negative bacteria. In *Cowan and Steel's Manual for the Identification of Medical Bacteria* 3rd edn (pp. 94–164). Cambridge: University Press.

Barrow, P.A., Jones, M.A. & Thomson, N. (2010). *Salmonella*. In C.L. Gyles, J.F. Prescott, G. Songer & C.O. Thoen (Eds.). *Pathogenesis of Bacterial Infections in Animals* 4th edn (pp. 231–265). Ames: Wiley-Blackwell.

Bassolé, I.H.N., Lamien-Meda, A., Bayala, B., Tirogo, S., Franz, C., Novak, J., Nebié, R.C. & Dicko, M.H. (2010). Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. *Molecules*, 15, 7825–7839.

Bauer, A.W., Kirby, W.M., Sherris, J.C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45, 493–496.

Bäumler, A.J., Tsolis, R.M., Ficht, T.A. & Adams, L.G. (1998). Evolution of host adaptation in *Salmonella enterica*. *Infection and Immunity*, 66, 4579–4587.

Belco, L.B.K. (1985). *Contribution à l'Etude des Modes actuels et de l'Importance socioéconomique de l'Elevage de Pintade au Bénin* (Thèse d'Ingénieur agronome). Cotonou: Université Nationale du Bénin.

Bertrand, S., Dierick, K., Heylen, K., De Baere, T., Pochet, B., Robesyn, E., Lokietek, S., Meervenne, E.V., Imberechts, H., De Zutter, L. & Collard, J-M. (2010). Lessons learned from the management of a national outbreak of *Salmonella* Ohio linked to pork meat processing and distribution. *Journal of Food Protection*, 73, 529–534.

Bessin, R., Belem, A.M.G., Boussini, H., Compaore, Z. & Kaboret, Y. (1998). Enquête sur les causes des mortalités des pintadeaux au Burkina Faso. *Revue d' Elevage et de Médecine Vétérinaire des Pays Tropicaux*, 51, 87–93.

Boko, C.K. (2004). *Contribution à l'Amélioration de l'Elevage villageois de la Pintade locale dans le Département du Borgou (Nord-Est du Bénin)* (DES en Gestion des Ressources animales et végétales en Milieux tropicaux). Liège: Faculté de Médecine vétérinaire, Université de Liège.

Boko, C.K., Kpodekon, M.T., Dahouda, M., Marlier, D. & Mainil, J. (2012). Contraintes techniques et sanitaires de la production traditionnelle de pintade en Afrique subsaharienne. *Annales de Médecine Vétérinaire*, 156, 25–36.

Boko, C.K., Kpodekon, M.T., Farougou, S., Dahouda, M., Youssao, A.K.I., Aplogan, G.L., Zanou, J. & Mainil, J.G. (2011a). Farmer perceptions and pathological constraints in helmeted guinea fowl farming in the Borgou department in north-east Benin. *African Journal of Agricultural Research*, 6, 2348–2357.

Boko, C.K., Kpodekon, M.T., Farougou, S., Youssao, A.K.I. & Mainil, J.G. (2011b). *In vivo* assessment of the virulence of five *Salmonella enterica* serotypes isolated from helmeted guinea fowl (*Numida meleagris*) in Benin. *International Research Journal of Microbiology*, 2, 356–364.

Boqvist, S., Hansson, I., Nord, B., Jerselius, U., Hamilton, C., Wahlström, H., Noll, B., Tysen, E. & Engvall, A. (2003). *Salmonella* isolated from animals and feed production in Sweden between 1993 and 1997. *Acta Veterinaria Scandinavica*, 44, 181–197.

Bornert, G. (2000). Poultry without *Salmonellae*: myth or reality? *Revue de Médecine Vétérinaire*, 151, 1083–1094.

CDC (Center for Disease Control and Prevention). (2009). Division of foodborne, waterborne and environmental diseases: national *Salmonella* surveillance data. Retrieved November 14, 2011, from http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm

Chakravortty, D., Hansen-Wester, I. & Hensel, M. (2002). *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *The Journal of Experimental Medicine*, 195, 1155–1166.

China, B., Pirson, V. & Mainil, J. (1996). Typing of bovine attaching and effacing *Escherichia coli* by multiplex *in vitro* amplification of virulence-associated genes. *Applied and Environmental Microbiology*, 62, 3462–3465.

Coburn, B., Grassl, G.A. & Finlay, B.B. (2007). *Salmonella*, the host and disease: a brief review. *Immunology and Cell Biology*, 85, 112–118.

Cornelis, G.R. (2006). The type III secretion injectisome. *Nature Reviews Microbiology*, 4, 811–825.

Cortez, A.L.L., Carvalho, A.C.F.B., Ikuno, A.A., Bürger, K.P. & Vidal-Martins, A.M.C. (2006). Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex–PCR. *Research in Veterinary Science*, 81, 340–344.

Courtney, S., Mossoba, M.E., Hammack, T.S., Keys, C. & Al-Khaldi, S.F. (2006). Using PCR amplification to increase the confidence level of *Salmonella typhimurium* DNA microarray chip hybridization. *Molecular and Cellular Probes*, 20, 163–171.

Dahouda, M. (2003). *Elevage de la Pintade locale dans le Département du Borgou au Bénin: Comparaison des Caractéristiques de Production en Station et en Milieu rural* (DEA en Sciences vétérinaires). Liège: Faculté de Médecine vétérinaire, Université de Liège.

Darwin, K.H. & Miller, V.L. (1999). Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clinical Microbiology Reviews*, 12, 405–428.

Euzeby, J.P. (1997). Les salmonelles et les salmonelloses aviaires dues aux sérovars ubiquistes. *Revue de Médecine Vétérinaire*, 148, 61–76.

Gassama-Sow, A., Wane, A.A., Canu, N.A., Uzzau, S., Aidara-Kane, A. & Rubino, S. (2006). Characterization of virulence factors in the newly described *Salmonella enterica* serotype Keurmassar emerging in Senegal (sub-Saharan Africa). *Epidemiology and Infection*, 134, 741–743.

Gast, R.K. (2008). Paratyphoid infections. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. Mcdougald, L.K. Nolan & D.E. Swayne (Eds.). *Diseases of Poultry* 12th edn (pp. 636–665). Ames: Blackwell Publishing.

Grimont, P.A.D. & Weill, F-X. (2007). *Antigenic formulae of the* Salmonella *serovars* 9th edn. Paris: Pasteur Institute World Health Organization Collaborating Centre for Reference and Research on *Salmonella*. Retrieved March 30, 2011, from http://www.pasteur.fr/sante/clre/cadrecnr/salmoms-index.html

Hensel, M. (2004). Evolution of pathogenicity islands of *Salmonella enterica*. *International Journal of Medical Microbiology*, 294, 95–102.

HPA (Health Protection Agency). (2012). Health protection reports: enteric infections. Retrieved August 14, 2012, from http://www.hpa.org.uk/hpr/infections/enteric.htm

Hughes, L.A., Shopland, S., Wigley, P., Bradon, H., Leatherbarrow, A.H., Williams, N.J., Bennett, M., de Pinna, E, Lawson, B., Cunningham, A. & Chantrey, J. (2008). Characterisation of *Salmonella enterica* serotype Typhimurium isolates from wild birds in northern England from 2005 – 2006. *BMC Veterinary Research*, 4, 1–10.

Hunter, S.B., Vauterin, P., Lambert-Fair, M.A., Van Duyne, M.S., Kubota, K., Graves, L., Wrigley, D., Barrett, T. & Ribot, E. (2005). Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. *Journal of Clinical Microbiology*, 43, 1045–1050.

ISO (International Organization for Standardisation). (2002). *Microbiology of food and animal feeding stuffs. Horizontal method for the detection of Salmonella spp. ISO 6579* 4th edn. Retrieved

February 24, 2004, from http://www.iso.org/iso/home/store/catalogue_ics.htm

Le Minor, S., Le Coueffic, E., Le Priol, A., Lefèvre, M., Ducilassin, M., Chippaux, C., Boche, R., Mered, B., Saliou, P. & Darrigol, J. (1975). Quinze nouveaux sérotypes de *Salmonella* du sous-genre I isolées en Afrique. *Bulletin de la Société de Pathologie Exotique*, 69, 547–555.

Malik-Kale, P., Jolly, C.E., Lathrop, S., Winfree, S., Luterbach, C. & Steele-Mortimer, O. (2011). *Salmonella* – at home in the host cell. *Frontiers in Microbiology*, 2, 125.

Marcus, S.L., Brumell, J.H., Pfeifer, C.G. & Finlay, B.B. (2000). *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes and Infection*, 2, 145–156.

Nakamura, C.V., Ueda-Nakamura, T., Bando, E., Melo, A.F., Cortez, D.A. & Dias Filho, B.P. (1999). Antibacterial activity of *Ocimum gratissimum* L. essential oil. *Memórias do Instituto Oswaldo Cruz*, 94, 675–678.

Oosterom, J. (1991). Epidemiological studies and proposed preventive measures in the fight against human salmonellosis. *International Journal of Food Microbiology*, 12, 41–52.

Quinn, P.J., Markey, B.K., Leonard, F.C., FitzPatrick, E.S., Fanning, S. & Hartigan, P.J. (2011). *Enterobacteriaceae.* In *Veterinary Microbiology and Microbial Disease* 2nd edn (pp. 263–286). London: Wolfe Publishing.

Schmidt, H. & Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clinical Microbiology Reviews*, 17, 14–56.

Shivaprasad, H.L. & Barrow, P.A. (2008). Pullorum disease and fowl typhoid. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. Mcdougald, L.K. Nolan & D.E. Swayne (Eds.). *Diseases of Poultry* 12th edn (pp. 620–630). Ames: Blackwell Publishing.

Skyberg, J.A., Logue, C.M. & Nolan, L.K. (2006). Virulence genotyping of *Salmonella* spp. with multiplex PCR. *Avian Diseases*, 50, 77–81.

Soussy, C.J. ed., (2010). Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM): Recommandations 2010. Paris: Société Française de Microbiologie. Retrieved June 10, 2004, from http://www.sfm.asso.fr/

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33, 2233–2239.

Villate, D. (2001). *Maladies des volailles* 2nd edn. Paris: France Agricole.

Willcox, W.R., Lapage, S.P. & Holmes, B. (1980). A review of numerical methods in bacterial identification. *Antonie Van Leeuwenhoek*, 46, 233–299.