

Full Length Research Paper

# ***In vivo* assessment of the virulence of five *Salmonella enterica* serotypes isolated from helmeted guinea fowl (*Numida meleagris*) in Benin**

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October 05 August, 2011

The pathogenicity of five *Salmonella enterica* serotypes (Farakan, Kingston, Legon, Oakland, Teshie) isolated from guinea fowl in Benin was experimentally assessed by oral challenge of 25 groups of 18 two week-old keets using 0.5 ml of  $10^4$  to  $10^8$  CFU/ml of overnight broth culture. One additional unchallenged group formed a control group. Keets were observed twice daily for clinical signs until day 15 post-inoculation. Clinical signs were observed in all groups, except in keets inoculated with serotype Teshie at  $10^4$  CFU/ml. Clinical scores were higher ( $p < 0.05$ ) at the highest inoculum concentrations within each serotype, but also differed between serotypes. Seven deaths were recorded only in serotypes Legon and Oakland. *Salmonella* was isolated from faeces with all serotypes at D7 and/or D15 PI at inoculum concentrations of  $10^6$ ,  $10^7$  and/or  $10^8$  CFU/ml. *Salmonella* was also recovered at D15 PI from the caecum of two keets of each group at  $10^7$  and  $10^8$  CFU/ml (except serotype Teshie), but not from the heart blood; there was only one from the liver. These five *Salmonella* serotypes are not only able to colonize keet intestines but also cause clinical signs similar, though less dramatic to those observed during field surveys.

**Keywords:** *Salmonella enterica*, serotypes, guinea fowl, oral challenge virulence, morbidity, mortality, packed cell volume, faecal excretion.

## INTRODUCTION

In the Borgou Department of North-East Benin, the raising of helmeted guinea fowl (*Numida meleagris*) is an important part of rural farming, both for farmers' own consumption, and for sale for various cultural and social uses (Branckaert and Gueye, 1999). Traditional guinea fowl breeding has for decades been based on the scavenging system, in which young keets roam freely around the coops mixing with the young and adults of different poultry species, all feeding predominantly on local flora and fauna. This raising system inevitably comes up against numerous sanitary constraints. Not surprisingly the death rate of keets within the first two months of life varies between 65 and 70% as a

consequence of poor and unhygienic housing and feeding conditions, as well as infectious and parasitic diseases (Boko, 2004; Sonaiya and Swan, 2004; Dahouda et al., 2007; Boko et al., 2011).

Several bacterial species have been isolated from dead keets during different surveys, but (with the exception of *Salmonella enterica*) they are not considered primary pathogens. Instead, they mostly represent members of the resident commensal microbiota, which invade the body around or after death, or secondary pathogens (*Escherichia*, *Klebsiella*, *Enterobacter*) benefiting from favourable conditions (stress, weakening, cold, viral infection, heavy parasitism) (Bessin et al., 1998; Boko, 2004; Boko et al., 2011).

*S. enterica* is subdivided into 6 subspecies and more than 2500 serotypes with different host range specificity (Tindall et al., 2005; Grimont and Weill, 2007). The

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poultry specific serotype Gallinarum/Pullorum has been reported worldwide, including in sub-Saharan countries, causing major economic losses in commercial poultry flocks as a consequence of high rates of morbidity and mortality of chicks during the first days of life (Pope, 2000; Villate, 2001; MAEP, 2005; Shivaprasad and Barrow, 2008). The Gallinarum/Pullorum serotype has also been described in guinea fowl in Europe (Galor, 1990; Le Coz-Doin, 1992; Shivaprasad and Barrow, 2008). In Benin, a sero-prevalence of 22% was reported in guinea fowl in a study conducted almost 20 years ago (Chrysostome et al., 1993) but unfortunately no more recent data are available. During a wide-scale survey in 2007/2008 in the rural area of Borgou, different *S. enterica* serotypes were isolated from 15% of dead keets, but none belonged to the Gallinarum/Pullorum serotype. Instead the *Salmonella* isolates belonged to different "exotic" serotypes, the most frequent being Farakan, Kingston, Legon and Oakland (Boko et al., 2008).

The objective of the present investigation was to assess the virulence of those "exotic" *Salmonella* serotypes by experimental infections of two week-old keets, using the oral route.

## MATERIALS AND METHODS

### Experimental animals

A total of 468 keets hatched from local guinea fowl eggs were purchased from five farms in the Borgou Department of North-East Benin. The eggs were incubated at the Production and Animal Health Department Research Farm of the Polytechnic School of Abomey-Calavi (Benin). At one-day-old the keets were distributed between 26 groups, each containing 18 individuals. Each group was kept in a separate pen within the same building. The keets were raised under strict hygiene and heat conditions. Local commercial antibiotic-free food and water were provided *ad libitum*. They were given an anticoccidial (Amprolium 20%, LAPROVET, France) from 8 to 12 days of age. At 14 days of age, they were de-wormed with an oral anthelmintic powder of pure piperazine citrate (LAPROVET, France).

### Bacterial isolates

Challenge strains were confirmed *S. enterica* strains isolated from dead rural keets in 2007 or 2008 and belonging to five different serotypes: Farakan, Kingston, Legon, Oakland, and Teshie (Boko et al., 2008). Sereotyping of *S. enterica* strains was carried out by slide agglutination with commercial monospecific antisera following the Kauffmann-White scheme (Grimont and Weill, 2007). It was performed at the Veterinary and Agrochemical Research Centre, Brussels, Belgium (Dr

Hein Imberechts) and confirmed at the *Salmonella-Shigella* National Reference Centre of the Scientific Institute of Public Health, Brussels, Belgium (Dr Sophie Bertrand).

Each strain was cultivated on *Salmonella-Shigella* agar (Oxoid, England) and incubated at 37°C overnight. Isolated colonies were transferred using sterile swabs into 10 ml sterile Laury Broth base (LB broth, Acros, Belgium) until reaching the optical density of 0.8 at 560 nm (water analysis spectrophotometer DR/2500, Orchidis, France) corresponding to the value of  $10^8$  Colony Forming Units (CFU)/ml. The bacterial suspension was then 10-fold serially diluted in LB broth to the corresponding value of  $10^4$  CFU/ml.

### Experimental challenge and keet surveillance

At 15 days of age, the 18 keets in each of the first 25 groups were orally inoculated with 0.5 ml of one dilution of one serotype, i.e. 90 keets per serotype. One group remained non-inoculated (negative control group) and received 0.5 ml of sterile LB broth. All animal experiments were carried out according to Ethical and Animal Welfare Regulation of the Polytechnic School of Abomey Calavi (University of Abomey Calavi, Benin).

All keets were observed twice daily for clinical signs (prostration, chills, somnolence, lack of appetite, whitish diarrhoea, arthritis, respiratory distress) and fatality cases, until 15 days post-inoculation (PI). Any clinical sign observed in any one animal was recorded as a positive observation. So in theory as many as 540 positive observations (2 daily observations x 18 animals x 15 days) were possible per group. Haematocrit, or Packed Cell Volume (PCV) was the only blood parameter measured at days (D) 0, D7 and D15 PI following standard procedure. One drop of blood was collected from the wing vein and centrifuged with a micro-haematocrit centrifuge (HAWKSLEY, England).

Fatality cases were recorded and necropsied. Moreover at D15 PI, two of the surviving keets from each group were randomly chosen, killed by cervical dislocation and necropsied

### Bacteriological analysis

*S. enterica* faecal excretion was estimated by collecting about 3g of faeces from each pen on D0, D7, and D15 PI. 1 g was subsequently suspended in 9 ml of sterile LB broth after thorough mixing (Lab Blender, Model 400, Seward Medical, London, UK) for 30 seconds (Moro Valliente, 2007). Heart blood, caecal content and liver samples were collected during necropsy of dead and sacrificed keets for isolation of *S. enterica*. The caecum and the liver were removed with a sterile scalpel, and 1 g was weighed and placed into a stomacher bag (Seward,

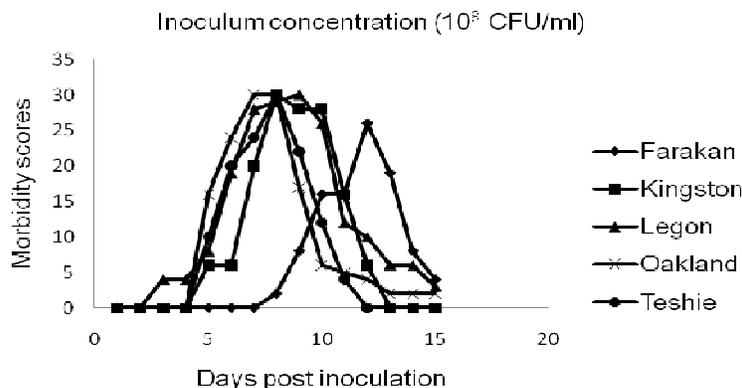


Figure 1a. Inoculum concentration of  $10^8$  CFU/ml.

Stomacher lab system, circulator 400, UK) containing 9 ml of sterile LB broth. The samples were then mixed (Lab Blender, Model 400, Seward Medical, London, UK) for 1 min before being 10-fold diluted. 0.1ml of each dilution was streaked onto two ready-to-use Xylose Lysine Desoxycholate (XLD) plates (Oxoid, UK) and incubated at 37°C for 24 - 48 h in order to perform *Salmonella* counts (Quinn et al., 1999; Bauwens et al., 2006). Only plates that yielded between 30 and 300 colonies were recorded.

One drop of heart blood was collected at necropsy following sterile procedure and streaked onto two 5% sheep blood Columbia and two MacConkey's agar plates (Oxoid, UK) prepared from dehydrated powders at the Production and Animal Health Department Laboratory of the Polytechnic School of Abomey-Calavi (Benin).

Three presumptive *Salmonella* colonies from each plate were transferred onto nutrient agar (Nutrient Medium, Oxoid, England), grown overnight at 37°C, and tested with urea broth, indol broth and Triple Sugar Iron (TSI) agar slant (Oxoid, England) tests for identification. All colonies with *Salmonella*-compatible test results (urea-negative, indol-negative, TSI with red slant surface, yellow bottom and H<sub>2</sub>S production) were sub-cultured overnight at 37°C on nutrient agar before performing rapid slide agglutination using OMA (Kingston, Legon), OMB (Oakland), OMC(Farakan) antisera (Bio-Rad, France).

### Statistical analysis

The statistical analyses were performed using the Statistical Analysis System (S.A.S) (1991). The procedure Proc. freq of SAS (1991) was used to calculate keet morbidity scores. A chi-square test was used to compare keet morbidity scores according to the inoculum concentrations and to the *Salmonella* serotypes. Additionally, a bilateral z-test was used to compare, two

by two, the morbidity scores for each concentration and each serotype.

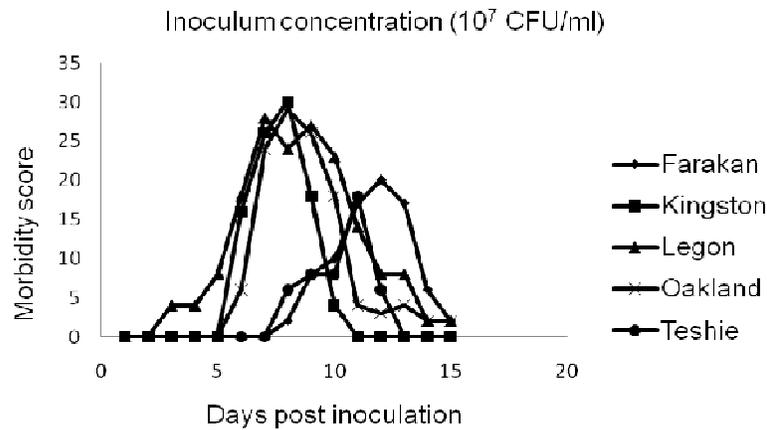
Finally, the serotype, the concentration and the days post inoculation were recorded as factors of variation of the haematocrit values. The PCV data were analysed according to General Linear Model procedure (GLM) of SAS (1991). Then, average PCV values were calculated per group of inoculated keets according to sampling day and compared two by two by using student's test.

## RESULTS

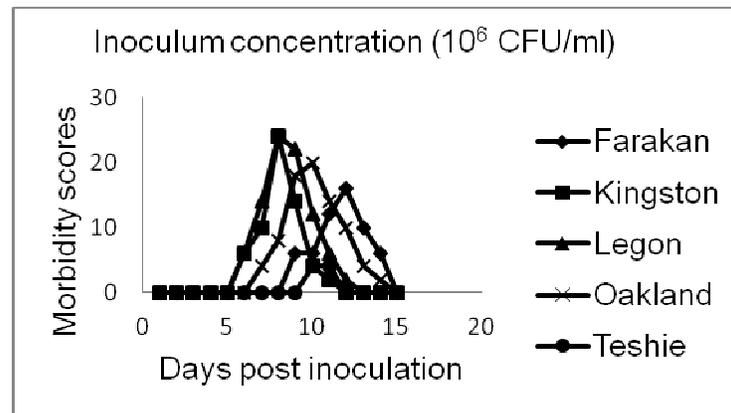
### Morbidity and mortality score

Clinical signs were observed in all groups of inoculated keets, with the exception of keets inoculated with serotype Teshie at the lowest concentration ( $10^4$  CFU/ml). However, signs observed were qualitatively and quantitatively different according to the serotype (Table 1a) and to the inoculum concentration (Table 1b). The highest morbidity score were recorded with serotype Legon at the highest four concentrations ( $10^5$  to  $10^8$  CFU/ml), and the lowest with the serotype Teshie at the lowest four concentrations of the inoculum ( $10^4$  to  $10^7$  CFU/ml) (Tables 1a and 1b; Figures 1a-e). Mortality was recorded with only two serotypes between days D5 to D7 PI: serotype Legon with 2 and 3 dead keets at the inoculum concentrations of  $10^7$  and  $10^8$  CFU/ml respectively, and serotype Oakland with 2 dead keets at the inoculum concentration of  $10^8$  CFU/ml. Neither morbidity, nor mortality was observed in the control group before the end of trial.

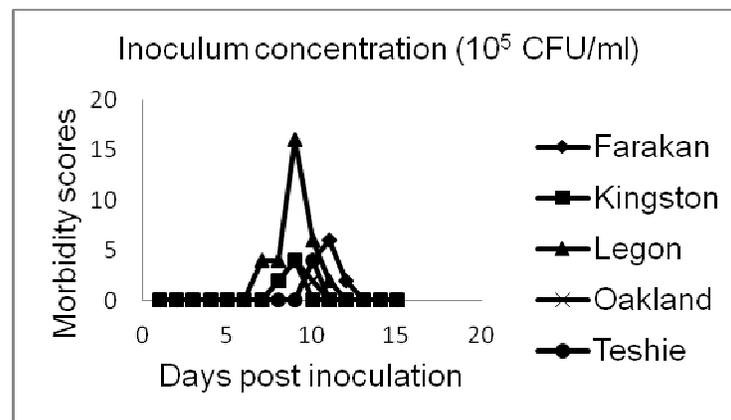
For all serotypes, clinical signs started between D2 and D7 PI at the highest two concentrations of the inoculum ( $10^7$  and  $10^8$  CFU/ml; Figs. 1a and 1b), and started between D5 and D9 PI at the inoculum concentrations of  $10^5$  and  $10^6$  CFU/ml (Figures 1c and 1d). Their magnitude (peak daily scores between 17-30) and duration (between 5 and 13 days) were very similar, if not



**Figure 1b.** Inoculum concentration of  $10^7$  CFU/ml.



**Figure 1c.** Inoculum concentration of  $10^6$  CFU/ml.



**Figure 1d.** Inoculum concentration of  $10^5$  CFU/ml.

identical, at the highest two concentrations, but were lower and/or shorter at the concentrations of  $10^5$  and  $10^6$  CFU/ml (peak daily scores between 4-26; duration between 1 and 8 days). At the lowest inoculum

concentration ( $10^4$  CFU/ml) of serotypes Farakan, Kingston and Legon, clinical signs appeared at D8 or D9 PI, lasting for 1 to 3 days and reaching a peak score of between 2 and 4 (Figure 1e). It is also noteworthy that

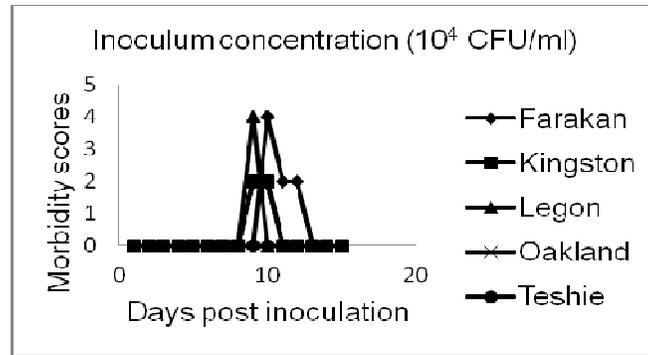


Figure 1e. Inoculum concentration of  $10^4$  CFU/ml.

Table 1a. Statistical significance by serotype.

Inoculum concentration	Morbidity score (%) *				
	$10^4$	$10^5$	$10^6$	$10^7$	$10^8$
Serotypes	CFU/ml	CFU/ml	CFU/ml	CFU/ml	CFU/ml
Farakan	1.48 <sup>a</sup>	2.22 <sup>b</sup>	10.37 <sup>c</sup>	15.18 <sup>c</sup>	18.33 <sup>c</sup>
Kingston	0.74 <sup>a</sup>	1.11 <sup>b</sup>	11.11 <sup>bc</sup>	17.40 <sup>bc</sup>	25.92 <sup>b</sup>
Legon	0.74 <sup>a</sup>	5.93 <sup>a</sup>	15.93 <sup>a</sup>	33.07 <sup>a</sup>	36.93 <sup>a</sup>
Oakland	0.74 <sup>a</sup>	1.48 <sup>b</sup>	14.81 <sup>ab</sup>	21.85 <sup>b</sup>	27.16 <sup>b</sup>
Teshie	0	0.74 <sup>b</sup>	1.48 <sup>d</sup>	8.51 <sup>d</sup>	22.59 <sup>bc</sup>

\*Maximum score 540 (see Materials and methods); except serotype Legon at inoculum concentration  $10^7$  CFU/ml (maximum score 514) and at inoculum concentration  $10^8$  CFU/ml (maximum score 501), and serotype Oakland at inoculum concentration  $10^8$  CFU/ml: maximum score 508), due to early deaths. <sup>a,b,c,d</sup> : Morbidity scores in the same column with different superscripts differed significantly ( $p < 0.05$ ).

Table 1b. Statistical significance by inoculum concentration.

Inoculum concentration	Morbidity score (%) *				
	$10^4$	$10^5$	$10^6$	$10^7$	$10^8$
Serotypes	CFU/ml	CFU/ml	CFU/ml	CFU/ml	CFU/ml
Farakan	1.48 <sup>c</sup>	2.22 <sup>c</sup>	10.37 <sup>b</sup>	15.18 <sup>a</sup>	18.33 <sup>a</sup>
Kingston	0.74 <sup>d</sup>	1.11 <sup>d</sup>	11.11 <sup>c</sup>	17.40 <sup>b</sup>	25.92 <sup>a</sup>
Legon	0.74 <sup>d</sup>	5.93 <sup>c</sup>	15.93 <sup>b</sup>	33.07 <sup>a</sup>	36.93 <sup>a</sup>
Oakland	0.74 <sup>d</sup>	1.48 <sup>d</sup>	14.81 <sup>c</sup>	21.85 <sup>b</sup>	27.16 <sup>a</sup>
Teshie	0 <sup>d</sup>	0.74 <sup>c</sup>	1.48 <sup>c</sup>	8.51 <sup>b</sup>	22.59 <sup>a</sup>

\* Maximum score of 540 (see Materials and methods); except serotype Legon at inoculum concentration  $10^7$  CFU/ml (maximum score 514) and at inoculum concentration  $10^8$  CFU/ml (maximum score 501), and serotype Oakland at inoculum concentration  $10^8$  CFU/ml: maximum score 508), due to early deaths. <sup>a,b,c,d</sup> Morbidity scores in the same line with different superscripts differed significantly ( $p < 0.05$ ).

clinical signs with serotype Farakan began and peaked later than with the other serotypes at all inoculum concentrations.

After reaching their respective peak scores, morbidity scores progressively decreased in all groups. At D15 PI, surviving keets were clinically healthy at all inoculum concentrations with the serotypes Kingston and Teshie, and at the lowest three inoculum concentrations ( $10^4$ ,  $10^5$ , and  $10^6$  CFU/ml) with serotypes Farakan, Legon, Oakland (Figures 1a-e).

## PCV values

The average PCV values of the control group were stable at D0, D7 and D15 PI, and decreased at D7 PI with all serotypes at all concentrations. The average PCV values rose again at D15 PI with serotypes Legon, Oakland and Kingston, but continued to decrease with serotypes Farakan and Teshie at all concentrations (Tables 2a, b, c). Nevertheless, only the PCV values at D7 and D15 PI of the groups inoculated with all five serotypes at the

**Table 2.** Statistical comparison of average PCV values per group of inoculated keets according to sampling day. The average PCV values (PCV %) and standard deviation (SD) of the non-inoculated group were respectively: D0 (41.11<sup>a</sup>, 0.75), D7 (41.05<sup>a</sup>, 0.72), D15 (41.27<sup>a</sup>, 0.66).

Inoculum Serotype	D PI	10 <sup>4</sup> CFU			10 <sup>5</sup> CFU			10 <sup>6</sup> CFU			10 <sup>7</sup> CFU			10 <sup>8</sup> CFU		
		PCV (%)	SD	PS	PCV (%)	SD	PS									
Farakan	0	41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8										
	7	39.61 <sup>a</sup>	0.5	NS	39.33 <sup>a</sup>	0.48	NS	39.11 <sup>a</sup>	0.47	NS	39.16 <sup>b</sup>	0.38	***	38.00 <sup>b</sup>	0.97	***
	15	39.66 <sup>a</sup>	0.48		39.61 <sup>a</sup>	0.5		39.61 <sup>a</sup>	0.5		36.77 <sup>c</sup>	0.64		36.11 <sup>c</sup>	0.96	
Kingston	0	41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8										
	7	39.61 <sup>a</sup>	0.5	NS	39.27 <sup>a</sup>	0.57	NS	38.50 <sup>a</sup>	0.7	NS	36.38 <sup>b</sup>	1.19	***	36.88 <sup>b</sup>	0.9	***
	15	39.72 <sup>a</sup>	0.46		39.61 <sup>a</sup>	0.5		39.61 <sup>a</sup>	0.5		39.11 <sup>c</sup>	0.83		38.72 <sup>c</sup>	0.66	
Legon	0	41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.57		41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8	
	7	39.72 <sup>a</sup>	0.46	NS	39.40 <sup>a</sup>	0.5	NS	38.61 <sup>a</sup>	0.69	NS	35.77 <sup>b</sup>	1.35	***	34.44 <sup>b</sup>	0.7	***
	15	39.72 <sup>a</sup>	0.46		39.61 <sup>a</sup>	0.5		39.61 <sup>a</sup>	0.5		39.18 <sup>c</sup>	0.83		38.53 <sup>c</sup>	0.51	
Oakland	0	41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.72		41.05 <sup>a</sup>	0.83		41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8	
	7	39.66 <sup>a</sup>	0.48	NS	39.33 <sup>a</sup>	0.48	NS	38.77 <sup>a</sup>	0.54	NS	36.66 <sup>b</sup>	1.41	***	35.77 <sup>b</sup>	1.16	***
	15	39.72 <sup>a</sup>	0.46		39.61 <sup>a</sup>	0.5		39.61 <sup>a</sup>	0.5		39.44 <sup>c</sup>	0.61		38.64 <sup>c</sup>	0.49	
Teshie	0	41.05 <sup>a</sup>	0.8		41.05 <sup>a</sup>	0.8										
	7	39.61 <sup>a</sup>	0.5	NS	39.33 <sup>a</sup>	0.48	NS	39.11 <sup>a</sup>	0.47	NS	39.16 <sup>b</sup>	0.38	***	38.00 <sup>b</sup>	0.97	***
	15	39.66 <sup>a</sup>	0.48		39.61 <sup>a</sup>	0.5		39.61 <sup>a</sup>	0.5		36.72 <sup>c</sup>	0.75		36.05 <sup>c</sup>	1.05	

a, b, c, : Average PCV values in the same column with different superscripts differed significantly ( $P < 0.001$ ); D PI: Days Post Inoculation; PCV: Packed Cell Volume; SD: Standard Deviation; PS: Percentage Significance; NS: Not Significant.

highest concentrations ( $10^7$  and  $10^8$  CFU/ml) were significantly different from the respective D0 and from the control group values ( $p < 0.001$ ).

#### Isolation of *Salmonella* from clinical samples and gross lesions

The faecal excretion tested positive for *Salmonella sp.* ( $10^3$  to  $3.10^5$  CFU/g) with all serotypes at D7 and/or D15 PI with inoculum concentrations of  $10^6$ ,  $10^7$  and/or  $10^8$  CFU/ml (Table 3). At necropsy, a slightly swollen caecum was observed in the five keets dead at D5 to D7

PI, but no apparent lesion was observed in any sacrificed keet at D15 PI.

*Salmonella sp.* was also isolated from the caecal contents of the 7 dead keets at D5 to D7 PI ( $2.10^7$  to  $3.10^7$  CFU/g) and that of the keets sacrificed at D15 PI ( $10^2$  to  $10^6$  CFU/g), inoculated with serotypes Farakan, Kingston, Legon, and Oakland, at the two highest concentrations ( $10^7$  and/or  $10^8$  CFU/ml; Table 4). *Salmonella sp.* was also isolated from the liver sample (50 CFU/g) of one keet inoculated with the serotype Farakan at the concentration of  $10^8$  CFU/ml. Conversely no *Salmonella sp.* was isolated from any heart blood sample.

#### DISCUSSION

Though *S. enterica* is usually regarded as a primary pathogen in animals, the intensity of the clinical signs and lesions is often dependent on the serotype of the *Salmonella* strain and on adjuvant infectious (parasitism, viral infection) or non-infectious circumstances (diet, stress etc.), particularly in birds (Le Coz Doiun, 1992; Villate, 2001). For instance, the most pathogenic serotypes in birds are Gallinarum/Pullorum, Typhimurium, Typhimurium var. Copenhagen, Enteritidis etc. (Euzebey, 1997; Bornert G. 2000; Villate, 2001). The five serotypes in this

**Table 3.** *Salmonella* excretion (CFU/g) in keet faeces at D0, D7 and D15 PI. No faecal sample from the non-inoculated group tested positive.

Serotypes	D PI	Inoculum concentration (CFU/ml)				
		10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	10 <sup>8</sup>
Farakan	0	0	0	0	0	0
	7	0	0	0	10 <sup>5</sup> CFU/g	1.5 10 <sup>5</sup> CFU/g
	15	0	0	0	2 10 <sup>5</sup> CFU/g	3 10 <sup>5</sup> CFU/g
Kingston	0	0	0	0	0	0
	7	0	0	10 <sup>3</sup> CFU/g	2 10 <sup>3</sup> CFU/g	3 10 <sup>3</sup> CFU/g
	15	0	0	0	10 <sup>3</sup> CFU/g	10 <sup>3</sup> CFU/g
Legon	0	0	0	0	0	0
	7	0	0	1.2 10 <sup>4</sup> CFU/g	2 10 <sup>4</sup> CFU/g	3 10 <sup>4</sup> CFU/g
	15	0	0	0	10 <sup>3</sup> CFU/g	10 <sup>3</sup> CFU/g
Oakland	0	0	0	0	0	0
	7	0	0	10 <sup>3</sup> CFU/g	1.2 10 <sup>4</sup> CFU/g	1.4 10 <sup>4</sup> CFU/g
	15	0	0	0	0	10 <sup>3</sup> CFU/g
Teshie	0	0	0	0	0	0
	7	0	0	0	0	10 <sup>3</sup> CFU/g
	15	0	0	0	0	0

D PI: Days Post Inoculation; CFU/g: Colony Format Units of *Salmonella* per gram of faeces.

**Table 4.** *Salmonella* isolation per gram of caecal content of the two keets sacrificed at D15 PI according to serotype and inoculum concentration.

Group	Inoculum concentration (CFU/ml)	<i>Salmonella</i> counts (CFU/g of caecal content)				
		Farakan	Kingston	Legon	Oakland	Teshie
Control	None	0	0	0	0	0
	10 <sup>4</sup>	0	0	0	0	0
	10 <sup>5</sup>	0	0	0	0	0
	10 <sup>6</sup>	0	0	0	0	0
Infected	10 <sup>7</sup>	a) 4 10 <sup>5</sup> b) 3 10 <sup>5</sup>	0	a) 3 10 <sup>5</sup> b) 3.5 10 <sup>5</sup>	a) 3.5 10 <sup>4</sup> b) 5 10 <sup>4</sup>	0
	10 <sup>8</sup>	a) 1.5 10 <sup>6</sup> b) 10 <sup>6</sup>	a) 10 <sup>2</sup> b) 2 10 <sup>2</sup>	a) 5 10 <sup>5</sup> b) 4 10 <sup>5</sup>	a) 8 10 <sup>4</sup> b) 7.5 10 <sup>4</sup>	0

a) b) : animal identification.

trial are very rare, both in developed and in developing countries, and equally in mammals (including man) and birds (Le Minor et al., 1975; Boqvist et al., 2003; HPA, 2006). Moreover, their actual virulence is unknown. Therefore, in the present study, keets were experimentally inoculated in order to assess the potential virulence of these serotypes.

The clinical signs recorded after oral inoculation of 5.10<sup>3</sup> to 5.10<sup>7</sup> CFU are similar to, though less dramatic than, those observed in keets during a field survey in Benin (Boko et al., 2011). They are also similar to the clinical signs observed in commercial guinea fowl in France during a salmonellosis outbreak caused by *Salmonella* Gallinarum (Le Coz Doin, 1992), and to those reproduced in one-day-old white Leghorn chicks

inoculated with 5.10<sup>7</sup> CFU of serotype Enteritidis strain BN92R1, isolated from guinea fowl in France (Millemann et al., 2005). The clinical signs can be associated with the colonization of keets by, and subsequent growth of, *Salmonella* strains, since they were observed neither in the non-inoculated control group, nor during the fifteen days of acclimation before inoculation.

Nevertheless, the intensity of the clinical signs (morbidity scores) significantly differ between the serotypes (with serotype Legon being the most pathogenic in terms of score and duration, and serotype Teshie being the least pathogenic) suggesting that they differ either by some virulence-associated properties, or by their relative adaptation to 2-week-old guinea fowl. The study of the different repertoires of virulence genes

located in mobile genetic elements such as plasmids, *spvC* gene (Okamoto et al., 2009), or *Salmonella* pathogenicity islands (SPI), *InvA*, *spiC*, *misL*, *pipD* gene present in SPI-1, SPI-2, SPI-3 and SPI-5 could help to explain these differences (Gassama-sow et al., 2006; Skyberg et al., 2006). Alternatively, morbidity scores might have been higher if one-day-old keets had been inoculated in place of older birds, as birds are most probably contaminated at that early age in field conditions. It is also noteworthy that the least pathogenic serotype Teshie had been isolated only once during the field study in contrast to the other serotypes which occurred more frequently (Boko et al., 2008). It is also important to note that the presence of maternal antibody prior to the inoculation could not be excluded since no antigen kits of these serotypes are available for serum control before inoculation, although at two weeks of age the antibody level should already have significantly decreased.

Not surprisingly, the morbidity scores are positively correlated to the inoculum concentration. In agreement with the morbidity scores, the intestinal colonization of the keets (evidenced by *Salmonella* isolation from faeces and caecal contents) differs according to the *Salmonella* serotype and is positively correlated to the inoculum concentration.

Nonetheless, only seven mortality cases were recorded at the highest two inoculum concentrations with serotypes Legon and Oakland. Death during salmonellosis is usually the consequence either of dehydration caused by intense enteritis and diarrhoea, or of invasion of the blood stream and internal organs (septicemia and polyserositis). Neither was observed during this experimental procedure, as illustrated by the absence of positive blood cultures and by only one positive liver culture. The observed low mortality score and invasive properties of the inoculated strains are confirmed by other published results on studies of one-day-old *Salmonella* enteritidis orally challenged chickens (Desmidt et al., 1997, Millemann *et al.*, 2005). These low mortality score may likewise offer similar explanations as for morbidity score (actual virulence properties, no full adaptation to the host), but may also reflect the importance of adjuvant circumstances in the development of clinical salmonellosis, such as those described in field surveys (Boko et al., 2011).

The average PCV values recorded before inoculation are similar to the values reported in literature in one-month-old healthy guinea fowl in India (Kundu et al., 1993) and eight-month-old healthy guinea fowl in Ghana (Awotwi and Boohene, 1992). The average PCV values decrease in infected keets during the first week PI specifically around the peak of morbidity scores in all infected groups. This anaemia is also linked to lack of appetite and reduced growth rate of diseased keets. Christensen et al. (1996) suggest that low haematocrits are linked to the presence of viable bacteria in liver and

caecal content during an experimental study of *Salmonella* Gallinarum orally inoculated chickens. Unfortunately no bacterial analysis was performed at D7 of the present study. Nevertheless, in agreement with the above mentioned hypothesis regarding lack of appetite and reduced growth rate, the increase of the average PCV values at D15 PI coincides with the progressive disappearance of the clinical signs, the improvement of appetite, and the decrease of *Salmonella* faecal excretion (i.e. the progressive recovery of surviving keets from the infection), during the second week PI. Additionally, although *Salmonella sp.* could still be isolated from the caecal contents, only one liver sample tested positive.

The results of this study confirm that, besides the classical avian serotypes, the five "exotic" serotypes of *Salmonella* isolated from dead keets in Benin are actually virulent and able to reproduce clinical infection in keets similar to, though less dramatic than, natural infections (Boko et al., 2011), with only a few recorded deaths. Some differences were also observed in the pathogenicity of these serotypes, particularly at lower inoculum concentrations, possibly reflecting difference in host adaptation. We may therefore conclude that adjuvant field circumstances (age of the keets, parasitism, cold, rain etc.) are of crucial importance in the development of the acute clinical syndrome. Both chronic salmonellosis and a passive carrier state (in the caecum and/or internal organs) can nevertheless develop in the young guinea fowl with the risk of environmental contamination (via faecal excretion) and egg contamination during laying, leading to early deaths of keets as well as embryos. There is also a potential risk for humans who consume raw eggs, since those serotypes Farakan, Legon, Oakland have also been isolated from humans, albeit very rarely and /or several years ago (Le Minor et al. 1975; HPA-, 2004, 2006).

## ACKNOWLEDGMENTS

The authors are grateful to the Republic of Benin and the Association of Universities of the Francophonie (AUF) for financial support of this study. We are also grateful to our Animal Husbandry Technicians, Aizoun Frédéric, Setchebè Kesaije, and Sessou Philipps for their assistance while performing the trial.

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