Molecular epidemiology of community-acquired invasive non-typhoidal *Salmonella* among children aged 2–29 months in rural Gambia and discovery of a new serovar, *Salmonella enterica* Dingiri

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Sixty-two invasive non-typhoidal *Salmonella* (NTS) isolates from children aged 2–29 months in rural Gambia were examined for serovar prevalence and antimicrobial susceptibility, and characterized using multilocus sequence typing (MLST) of seven genes, *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*. *Salmonella enterica* serovar Enteritidis was the most common serovar (80.6 %), followed by *S. enterica* serovar Typhimurium (8.0 %). Thirty-three per cent of the isolates were resistant to all eight antimicrobials tested, including ampicillin (74.2 %), cotrimoxazole (64.5 %) and tetracycline (63 %). A total of 40.3 % of the NTS cases had an initial clinical diagnosis of malaria, whilst 27.3 % had a diagnosis of clinical pneumonia and 18 % had a diagnosis of septicaemia. MLST of NTS resulted in ten different sequence types (STs), of which five were novel, representing five different NTS serovars. In general, STs were restricted to the same serovar. One type (ST11) encompassed 80.6 % of the NTSs. A new NTS serovar named *S. enterica* serovar Dingiri was discovered. *S. Dingiri* was isolated from a 6-month-old male with an initial clinical diagnosis of malaria but a final clinical diagnosis of anaemia and septicaemia. *S. Dingiri*, which possesses an antigenic formula of 17:z:1,6, was sensitive to ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, cotrimoxazole and tetracycline but resistant to gentamicin, and was ST338.

Received 26 May 2007

Accepted 23 July 2007

INTRODUCTION

Non-typhoidal *Salmonella* (NTS) is one of the most important enteric pathogens causing bacteraemia in young children in many parts of the world, including Africa (Hill *et al.*, 2007; Enwere *et al.*, 2006; Berkley *et al.*, 2005; Mulholland & Adegbola, 2005). Extraintestinal infections caused by *Salmonella* species are a common cause of severe morbidity and mortality among young children from tropical Africa (Hill *et al.*, 2007; Enwere *et al.*, 2006; Berkley *et al.*, 2005). In industrialized countries, NTS usually causes acute gastroenteritis, while extraintestinal infection is uncommon (Graham *et al.*, 2000a). In contrast, recent reports from sub-Saharan Africa, including The Gambia, show that NTS was consistently one of the most common

causes of paediatric bacteraemia (Hill *et al.*, 2007; Enwere *et al.*, 2006; Kariuki *et al.*, 2006). In the course of a pneumococcal-conjugate vaccine trial (PVT) in rural Gambia, during a period when *Haemophilus influenzae* type b had been virtually eliminated, Enwere *et al.* (2006) showed that NTS was the second most common blood culture isolate after *Streptococcus pneumoniae* in children with invasive bacterial disease. NTS infection is associated with malaria and anaemia, and this often confuses diagnosis and delays appropriate management (Graham *et al.*, 2000b; Berkley *et al.*, 1999). For example, NTS was the most common isolate from blood culture in Ghanaian children who remained febrile after clearance of malarial parasitaemia (Commey *et al.*, 1994). Global emergence of antibiotic resistant NTS has been described (Gebreyes & Thakur, 2005; Lee *et al.*, 2003; Kariuki *et al.*, 2006; Soler *et al.*, 2006). Surveillance of the distribution and antimicrobial susceptibility patterns of NTS serovars, and

Abbreviations: HIV, human immunodeficiency virus; MLST, multilocus sequence typing; NTS, non-typhoidal *Salmonella*; PVT, pneumococcal-conjugate vaccine trial; ST, sequence type.

molecular characterization of NTS isolates from sick children in sub-Saharan Africa are, however, scanty. Surveillance of NTS disease is important not only to establish the current, regional epidemiology of invasive NTS serovars, but also to monitor the emergence and prevalence of antimicrobial resistance, to detect the mutational changes that may occur over time in the serovars allelic profile, and to link these to changes in the pathogenesis of the commonest serovars.

METHODS

Study population. A total of 94 NTS isolates were obtained from 86 children who were investigated as possible cases of invasive bacterial infection during a PVT in rural Gambia (Enwere *et al.*, 2006). However, for unknown reasons, 35 NTS isolates could not be recovered after long-term storage, and hence were excluded from this particular study. Consequently, 59 of the 94 PVT NTS isolates were used in this study. Three additional NTS isolates were collected from children in the study area who were not enrolled in the PVT. Details of how children were enrolled, followed up and investigated have been described previously (Cutts *et al.*, 2005; Enwere *et al.*, 2006). Briefly, blood cultures were obtained from study children admitted to hospital if they had signs of pneumonia, meningitis or septicaemia, and from children managed as outpatients if they had signs of pneumonia and a temperature of ≥ 38 °C. Laboratory diagnosis of NTS was based on isolation of the bacterium from blood and/or cerebro-spinal fluid. Isolates were stored at -70 °C in 15 % glycerol broth before further testing. The study was approved by the Joint Gambia Government and Medical Research Council Ethics Committee, and ethics committees at the London School of Hygiene and Tropical Medicine and the National Institute of Health, USA.

Microbiological methods. NTS isolates were obtained as described above from patients who were investigated as possible cases of invasive bacterial infection between August 2000 and April 2004. NTS were isolated from blood using an automated blood-culturing system as previously described (Enwere *et al.*, 2006). NTS were identified using biochemical (bioMérieux analytical profile index, API 20 E) and agglutination (Bio-Stat *Salmonella* polyvalent agglutinating sera 'O' and 'H') methods. All strains were serotyped with antisera from the Statens Serum Institute. A total of 50 % of the isolates were sent to Statens Serum Institute for confirmation. When a new serovar was discovered this was sent to the Pasteur Institut, Paris, France, for evaluation.

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined by both disc diffusion and MIC methods. The following antimicrobial agents were tested by disc diffusion: ampicillin, cotrimoxazole, chloramphenicol, tetracycline, ciprofloxacin, cefotaxime, gentamicin and nalidixic acid (Oxoid). *S. Enteritidis* ATCC 13076 was used as a control and data were interpreted as susceptible or resistant according to the table in the publication by the NCCLS (2004). MIC of each isolate was tested by Etest strips (AB Biodisk) following the manufacturer's instructions. The following antimicrobial agents were tested by Etest: ampicillin, cotrimoxazole, tetracycline, chloramphenicol, cefotaxime and ciprofloxacin. The control strain *S. Enteritidis* ATCC 13076 was included in the test.

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Multilocus sequence typing (MLST). NTS isolates were streaked on blood agar and incubated at 37 °C for 18 h. A single colony from each

isolate was picked, restreaked and incubated at 37 °C for 18 h. Genomic DNA templates were prepared from a loopful of bacteria using a genomic DNA kit (Qiagen) according to the manufacturer's instructions. MLST was performed as described in the *Salmonella* MLST database (<http://web.mpiib-berlin.mpg.de/mlst>). Briefly, the seven genes targeted were *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*. Amplifications for all genes were carried out with approximately 0.2 µg DNA template, 250 µM each dNTP, 2.5 mM MgCl₂, 25 pmol primers and 1 U *Taq* polymerase (Qiagen) in a 25 µl reaction mixture. PCR cycling conditions were a 10 min hold at 94 °C, followed by 34 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Aliquots (2 µl) of reaction mixtures were separated by 1.0 % agarose gel electrophoresis, and visualized with ethidium bromide staining and UV illumination, and using a gel documentation system (Gel Doc 2000; Bio-Rad).

PCR products were sent to Macrogen (www.macrogen.com) for purification and DNA sequencing on both strands. Sequences were edited, and complementary sense and antisense fragments were aligned using Laser Gene DNASTar 7.1 software. The sequences were submitted to the MLST database website (<http://web.mpiib-berlin.mpg.de/mlst>), and assigned an existing or novel allele type number and sequence type (ST) number defined by the database. This multi-micro-organism database defines a novel allele type as a sequence containing one or more nucleotide changes from existing allele sequences. Composite STs are assigned based on the set of allele types derived from each of the seven loci. STs were analysed for relatedness using the eBURST v3 program (<http://eburst.mlst.net>). Cluster analysis of allelic profiles was performed by using a categorical coefficient and a graphic method called a minimum spanning tree with Bionumerics software (version 4.0; Applied Maths).

RESULTS AND DISCUSSION

This is believed to be the first study from The Gambia describing serovar prevalence and molecular characterization of NTS obtained from patients with an invasive infection. The mean age of the patients studied was 18 months (median 22 months, range 4–24 months) and initial clinical diagnoses (made prior to culture results) are shown in Table 1.

Nine different serovars (Table 2) were identified, including a new serovar, namely *Salmonella enterica* serovar Dingiri. *S. Dingiri* was isolated from a 6-month-old male with an initial clinical diagnosis of malaria, but a final clinical diagnosis of anaemia and septicaemia. *S. Dingiri*, which possesses an antigenic formula of 17:z:1,6, was sensitive to ampicillin, cefotaxime, chloramphenicol, ciprofloxacin, cotrimoxazole and tetracycline, but resistant to gentamicin.

The commonest serovar was *S. enterica* serovar Enteritidis (80.6 %), followed by *S. enterica* serovar Typhimurium (8 %). Others include *S. enterica* serovar Offa (*S. Offa*), *S. enterica* serovar Virchow (*S. Virchow*), *S. enterica* serovar Stanleyville (*S. Stanleyville*), *S. enterica* serovar Dublin (*S. Dublin*), *S. enterica* serovar Hull (*S. Hull*), *S. enterica* serovar Camberene (*S. Camberene*) and the new serotype *S. Dingiri*, for each of which one isolate was obtained. Data on the prevalence of NTS in Africa is limited, and different geographical regions harbour different serovars, and different serovars predominate at different periods of time

Table 1. Initial clinical diagnoses of cases of invasive NTS disease by serovar in 62 Gambian children aged 2–29 months

Initial clinical diagnosis	S. Enteritidis	S. Typhimurium	Other serovars	No. (%)
Malaria	23	1	S. Dingiri x1	25 (40.3)
ALRI	11	3	S. Offa x1; S. Hull x1; S. Dublin x1	17 (27.3)
Septicaemia	10	1	0	11 (18)
Gastroenteritis	4	0	0	4 (6.4)
Meningitis	1	0	S. Virchow x1	2 (3.2)
Malnutrition	0	0	S. Camberene x1; S. Stanleyville x1	2 (3.2)
Anaemia	1	0	0	1 (1.6)
Total	50 (80.6 %)	5 (8.1 %)	7 (11.3 %)	62 (100)

ALRI, Acute lower respiratory infection.

(Galanis *et al.*, 2006). Nevertheless, a World Health Organization survey in all age groups on the global distribution of *Salmonella* between 2000 and 2002 showed that among human isolates, *S. Enteritidis* was the most common serovar, accounting for 65 % of all isolates followed by *S. Typhimurium* at 12 % and *S. Newport* at 4 % (Galanis *et al.*, 2006). The global prevalence of NTS concurs with our observation in The Gambia within the same time period. However, in contrast to other parts of Africa (Cameroon, Mali, Morocco, Senegal and Tunisia) in 2002, *S. Enteritidis* and *S. Typhimurium* were each reported in equal proportion in all age groups (Galanis *et al.*, 2006). These differences in the pattern of NTS infections may be due to ecological (animal reservoirs) or geographical differences in the Africa subregion. Furthermore, there are also different criteria for investigation in different studies – our patients probably included a higher proportion with clinical pneumonia than most other studies, because pneumonia was the main trigger for doing a blood culture in our study. Other studies may have taken cultures from all children with bacteraemia, or gastroenteritis and bacteraemia (Kariuki *et al.*, 2006; Hill *et al.*, 2007).

Invasive NTS in children results in a high mortality, and rapid administration of appropriate antimicrobial therapy can be life-saving. The susceptibility pattern of NTS serovars in this study showed a high proportion susceptible to cefotaxime (100 %), ciprofloxacin (100 %), nalidixic acid (100 %), chloramphenicol (80.6 %) and gentamicin (54.8 %). Susceptibility to ampicillin (26 %), co-trimoxazole (35 %) and tetracycline (37 %) was much lower. It is encouraging that this study found that Gambian isolates of NTS are still largely sensitive to chloramphenicol, which remains a first line treatment for severe pneumonia, septicaemia and meningitis locally and is more affordable than cefotaxime or ciprofloxacin. In Malawi, NTS isolates are more resistant to chloramphenicol *in vitro* Walsh *et al.* (2000). In addition, Hadfield *et al.* (1985) and Lepage *et al.* (1984) have shown that multi-resistant *Salmonella* are widespread in Africa.

Interestingly, we observed that *S. Enteritidis* was less likely to be susceptible to commonly used antimicrobials than was *S. Typhimurium* (Fig. 1), although the number of *S. Typhimurium* isolates was low, so there is substantial uncertainty around the estimates of resistance for this

Table 2. Serovar distribution and STs of NTS isolates used in this study

Serovar	ST	No. of isolates	Allelic profile						
			<i>aroC</i>	<i>dnaN</i>	<i>hemD</i>	<i>hisD</i>	<i>purE</i>	<i>sucA</i>	<i>thrA</i>
S. Dublin	10	1	5	2	3	6	5	5	10
S. Enteritidis	11	50	5	2	3	7	6	6	11
S. Typhimurium	19	4	10	7	12	9	5	9	2
S. Typhimurium	34	1	10	19	12	9	5	9	2
S. Virchow	181	1	70	7	10	10	2	10	14
S. Dingiri	338	1	11	40	25	16	12	16	63
S. Stanleyville	339	1	17	7	35	9	5	9	14
S. Hull	340	1	17	58	3	76	66	10	12
S. Offa	341	1	50	14	18	78	64	6	63
S. Camberene	342	1	19	70	67	75	45	7	39

Fig. 1. Comparison of susceptibility pattern between *S. Enteritidis* (black bars) and *S. Typhimurium* (hatched bars).

serovar. The reason for the marked differences in the susceptibility pattern in *S. Enteritidis* and *S. Typhimurium* is not clear, but it could be that the plasmid-resistance genes that often confer resistance to multiple classes of antimicrobials are more widely spread in *S. Enteritidis* than *S. Typhimurium*, but this will require further investigation.

Comparisons were made between disc diffusion, MIC test and MIC agar dilution methods (data not shown) for assessing antimicrobial sensitivity. The results obtained from these methods concur. Our observations show that disc diffusion susceptibility method can be a reliable technique provided that experienced laboratory personnel conduct the test and strictly adhere to the test procedure with inclusion of appropriate controls. In a developing country like The Gambia, the disc diffusion method has advantages as a means of guiding treatment because it is inexpensive, easy to perform and the materials are readily available, in contrast to MIC test, Etest and agar dilution methods, which are very expensive and laborious.

MLST is now the method of choice for the detailed characterization of a number of bacterial pathogens (Aanensen & Spratt, 2005; Sukhnanand *et al.*, 2005; Torpdahl *et al.*, 2005; Urwin & Maiden, 2003). Although it has been reported that MLST of several housekeeping genes provides a satisfactory level of discrimination among diverse *Salmonella* isolates (Kotetishvili *et al.*, 2002), recent studies suggest that MLST may not be suitable for distinguishing closely related strains within a particular serovar, due to high sequence identity and the slow accumulation of variations in their housekeeping genes (Sukhnanand *et al.*, 2005). Torpdahl *et al.* (2005) presented MLST data on 25 serotypes of *S. enterica* using the same 7-gene MLST scheme used in this study, and found that, overall, discrimination was not improved within a serotype compared with PFGE and amplified fragment length polymorphism data. This study confirms that, in general, STs are restricted to the same serovar, especially in the case

of *S. Enteritidis*, and the results from our dataset (Table 1) and the *Salmonella* MLST database (<http://web.mpiib-berlin.mpg.de/mlst>) were concordant. Interestingly, we discovered five novel STs (Table 2) including: (a) an *S. Dingiri* novel ST (ST338), which shares only two alleles with human *S. Javiana* ST24, and *S. Saintpaul* ST126 isolates reported in the USA and *S. Oranienburg* ST174, found in snakes and lizards in Germany; (b) an *S. Stanleyville* ST, designated ST339, which is a tri-locus variant with *S. Typhimurium* ST19, ST98, ST99 and ST153, and *S. Thompson* ST25; (c) an *S. Hull* ST340, which shares two alleles with an unknown serovar in the MLST database and with ST202 found in sheep; (d) an *S. Offa* ST341, which shares three alleles with *S. Mbandaka* ST206 found in swine in Canada; (e) an *S. Camberene* ST342, which shares three alleles with *S. Senftenberg* ST218 found in China.

Furthermore, MLST results showed that *S. Typhimurium* exhibits two STs, namely ST19 and ST34. ST19 and ST34 are single locus variants at the *dnaN* locus, and sequence analysis showed that the different alleles at this locus (*dnaN* 7 and *dnaN* 19) differ at a single base pair (G to A), suggesting a spontaneous mutation event caused the change in allele type at this locus. As this is believed to be the first report characterizing NTS from developing countries using the seven genes selected for the global MLST *Salmonella* database housed at the Max-Planck Institut für Infektionsbiologie, Germany (<http://web.mpiib-berlin.mpg.de/mlst/>), further study using this scheme with a larger number of isolates and additional serovars from Africa is necessary to fully explore the utility of MLST for epidemiological purposes.

The association of NTS infection with a number of clinical conditions has been well documented. In The Gambia, Mabey *et al.* (1987), found that patients with NTS septicaemia had a significantly higher prevalence of malarial parasitaemia than did patients with other forms of septicaemia ($P < 0.01$). We observed that 40.3 % (25) of the total NTS cases were associated with an initial clinical diagnosis of malaria, whilst 27.3 % were associated with acute lower respiratory infection and 18 % with septicaemia (Table 1).

Human immunodeficiency virus (HIV) is another important predisposing factor for invasive NTS infections, although the reason for this is not clear. Published studies (Gilks *et al.*, 1990; Nathoo *et al.*, 1996) have demonstrated an association between HIV and bacterial infections, and Rongkavilit *et al.* (2000) have reported that in the USA and Europe *Salmonella* bacteraemia is commonly associated with childhood HIV infection. HIV status was not investigated in the children in our study but few are likely to have been HIV positive as the overall prevalence of HIV infection in the adult Gambian population is about 2 % (Schim van der Loeff *et al.*, 2003).

This study has confirmed previous findings from The Gambia (O'Dempsey *et al.*, 1994; Mabey *et al.*, 1987) and

from other parts of sub-Saharan Africa that NTS is an important cause of serious illness in young children. However, very little is known about the epidemiology of this infection, for example how the infection is transmitted to young children and whether invasive infections are recently acquired or represent a reactivation of a previously established, asymptomatic infection. The development of new tools to characterize NTS opens the way to more detailed epidemiological studies of this important infection.

ACKNOWLEDGEMENTS

We acknowledge the use of the *Salmonella* MLST database (<http://web.mpiib-berlin.mpg.de/mlst>), which is housed at Max Planck Institute, Berlin, Germany. The Gambia Pneumococcal Vaccine Trial was funded by grants from: the National Institute of Allergy and Infectious Diseases at the USA National Institutes of Health through contract no. 1-AI-25477; the World Health Organization through contract V23/181/127; the Children's Vaccine Program at PATH; and the USA Agency for International Development.

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