Can herbal remedies be the answer to multidrug resistance? 
Profile of drug resistance in Salmonella species in Eastern Cape, South Africa

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Summary

Objective: The treatment of serious Salmonella infections which requires the use of cephalosporins and fluoroquinolones is being compromised by the emergence of extended-spectrum beta-lactamases (ESBLs). This study reports the antibiotic profile of Salmonella species, highlighting increasing ESBLs trends in Salmonella spp. and the emergence of multi-drug resistance (MDR). To proffer solution to the problem of MDR, screening of selected herbal plants was carried out.

Methods: 142 consecutive isolates of Salmonella spp. collected over a period of 4 years were tested for antibiotic resistance. Antibiogram, ESBL phenotype and confirmation of isolate were determined using a semi-automated antibiotic test. Tests were performed based on Clinical Laboratory Standards Institute standards for broth microdilution methods and interpretation using Escherichia coli ATCC 25922 as the control strain. Antibiotic resistant patterns were determined, ranking order of importance as percent (%) of each type of resistance. Twelve plants selected based on ethnobotanical survey information as remedy in the treatment of stomach related ailments were screened using broth microdilution methods against strains of Salmonella, Shigella, Escherichia, Staphylococcus, Pseudomonas and Enterococcus.

Results: A greater proportion of isolates were obtained from invasive cultures. Of the Salmonella isolates, there was a striking predominance of S.enterica serotype Typhi followed by S.enterica serotype Typhimurium. Most species showed pentavalent resistance to commonly used drugs. Antimicrobial resistance in S.enterica serotype Typhi is visibly increasing. Of growing concern is the increase in strains exhibiting ESBLs. Plant screening revealed promising therapeutic values in Aloe arborescens, A.striatus, and Psidium guajava.

Conclusion: Increasing MDR in Salmonella serovars involved ESBLs’ production. Plants with significant antibacterial activities were comparable to the tested antibiotic giving credence to their use in ethnomedicine. With further isolation of bioactive components, these plants may be a relief to multidrug resistance enteric pathogens.

Key words: Antimicrobial; Drug resistance; ESBL; Herbal remedies; Salmonella

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Introduction

With the fraught of increasing HIV/AIDS infections in the sub-Saharan Africa, antibiotic resistance is of critical concern because of the serious complications which can ensue in immuno-compromised individuals even from the mildest form of enteric infections. Salmonellae are often implicated in food-borne outbreaks with S.enterica serovar Typhimurium and S.enterica serovar Enteritidis being the most frequently isolated serovars from such outbreaks throughout the world [1]. Studies have also linked diarrhoea cases due to Salmonella spp. and other enteric pathogens to water contamination [2, 3]. The clinical pictures of infections with Salmonella range from gastroenteritis, bacteraemia or septicemia, enteric fever to a carrier state in persons with previous infections [4]. Salmonella diseases such as typhoid, paratyphoid fevers and septicemia can often be fatal in hosts with impaired defences such as the elderly or patients with acquired immunodeficiency.
syndrome [5, 6]. The treatment of serious Salmonella infections requires the use of cephalosporins and fluoroquinolones. However, the emergence of extended-spectrum beta-lactamases (ESBLs) has led to their effectiveness being compromised [7, 8].

More than 80% of the population in developing countries of the world depends on plants for their medical needs [9, 10]. Of the South African population 60% consults one of an estimated 200,000 traditional healers, in preference to, or in addition to Western medical doctors, especially in rural areas [11].

Medicinal plants have been acknowledged as potential sources of new compounds of therapeutic value and as sources of lead compounds for drug design and development [12]. Furthermore, the emerging global problem of multidrug resistant pathogens [13, 14] and the need for the discovery of lasting and sustainable therapy to combat diseases such as HIV/AIDS, malaria and cancer which have defied available treatments has led to a paradigm shift to natural herbal product for succor. This study reports the antibiotic profile and rising multidrug resistance among Salmonella spp., highlighting the emergence of increasing ESBL trends, in addition to screening of some medicinal plants used in the treatment of diarrhoea and associated infections as probable alternate therapy.

Materials and methods

Ethical approval
Institutional ethical clearance of the Walter Sisulu University and the Eastern Cape province of South Africa’s Department of Health ethical approval were obtained before embarking on this study.

Bacteria identification and antibiogram
One hundred and forty-two (142) consecutive Salmonella isolates collected from patients attending Nelson Mandela Academic Teaching Hospital, a referral hospital in the Eastern Cape, South Africa, over a period of 4 years were selected for further analysis. These were retrieved from those deposited in the Enteric Diseases Reference Unit of the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. Antibiogram, minimum inhibitory concentration (MIC), ESBL phenotype determination and confirmation of Salmonella isolates identity were done with an automated antimicrobial susceptibility system (MicroScan, autoSCAN-4; Siemens). The identification and antibiotic susceptibility testing (ID/AST) was performed using the Dried Overnight Negative Combo panel 50 system which is made up of 23 antibiotics including β-lactams and extended-spectrum cephalosporins such as ceftazidime, cefotaxime and ceftriaxone. Tests were carried out according to the manufacturer’s instructions. The system and its interpretation are based on the Clinical and Laboratory Standards Institute (CLSI) standards for broth microdilution methods and interpretation [15]. Antibiotic resistant patterns were ranked in order of importance as percent (%) of each type of resistance for each sample evaluated. The serotyping was carried out by the slide agglutination method following the CDC laboratory protocol for serotyping of S. enterica O and H antigen [16] using commercially available Salmonella O- and H-antisera (BiowebSA). The definition of serotype was based on Kauffmann-White scheme [17].

Plant material extraction
Twelve herbs were selected based on the ethnosurvey information of their use in treatment of diarrhoea and stomach ailments [18]. The plants were collected from Port St. Johns, Flagstaff and Lusikisiki all in the Oliver R. Tambo District Municipality, Eastern Cape Province of South Africa. The selected plants (Table 1) were rinsed, dried and ground into fine powder out of which 50 g was macerated in 500 ml acetone. The choice of acetone as a solvent for the extraction of plant materials, was informed by its low toxicity to the tested pathogens among other criteria [19] and because it extracted a range of polar and non-polar compounds as previously described [20]. Extraction was carried out according to the method of Eloff [21].

TLC fingerprinting of extracts
Thin layer chromatography (TLC) was used to determine the composition of extracts. The TLC plates were prepared in duplicate and developed in different mobile solvent systems according to Eloff [20] using benzene:ethanol:ammonium hydroxide (BEA) (36:4:0.4); ethylacetate:methanol:water (EMW) (40:5.4:4); chloroform:ethylacetate:formic acid (CEF) (20:16:4). An aliquot of 10 µl of extract was separated by TLC (Merck Kieselgel 60 P254) in a closed, saturated TLC tank. Chromatograms were visualized under visible and ultraviolet light (254 nm and 360 nm, Camac Universal UV lamp TL-600). One TLC plate per solvent system was then sprayed with Vanillin-sulphuric acid (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) for the detection of higher alcohols, phenols, and steroids [22]. The plates were heated at 105°C until the colours of chromatograms were completely
developed. The $R_f$ value (retention factor) on TLC was used as the parameter for qualitative comparison. A further qualitative assignment is by the visualisation of native fluorescence of separated substances which is excited by UV (Ultraviolet) light at 254 nm and 366 nm but mostly long-wave UV.

**Plant antibacterial assay**

The MIC of extracts at a concentration of 10 mg/ml was determined by a serial broth microplate dilution technique as previously described [23]. ESBL-positive (ESBL+) *Salmonella enterica* serovar Typhimurium, ESBL-negative (ESBL-) *S.enterica* serovar Typhimurium, *Shigella flexneri* type 2a, and *Sh.sonnei* were selected among the clinical isolates previously screened with antibiotics. Typed culture of *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 21212), *Pseudomonas aeruginosa* (ATCC 25922), and *Staphylococcus aureus* (ATCC 29213), were included in the bioasay as control strains and for a broad-spectrum screening. Gentamicin (50 mg/ml), acetone and sterile Mueller-Hinton broth were the positive and negative antimicrobial controls respectively.

**Statistical analysis**

Data were analyzed using Microsoft Excel 2007. Values were expressed as mean and or ranked in order of importance as percent (%).
Results

Prevalence and susceptibility pattern of Salmonella species

The most prevalent of the eight typable serotypes were *S.enterica* serovar Typhi (53%); this was followed by *S.enterica* serovar Typhimurium (25%), *S.enterica* serovar Isangi (8.5%), *S.enterica* serovar Choleraesuis (5.6%), *S.enterica* serovar Enteritidis (2.8%), *S.enterica* serovar Eppendorf (2.1%), *S.enterica* serovar Hadar (1%), *S.enterica* serovar Panama (1%) and untypable strains (2.1%). *Salmonella* isolates were resistant to amoxicillin, ampicillin, aztreonam, piperacillin/tazobactam, trimethoprim/sulfamethoxazole and tetracycline with reduced susceptibility to ciprofloxacin. Resistance to 5 or more CLSI antibiotic subclasses was detected in 50% (71/142). Extended spectrum cephalosporins resistant phenotype was exhibited by 26% (37/142). There was a gradual increase in percent of isolates producing ESBLs from 16.2% to 37.8% over the 4 year period (Fig.1).

Plant extracts analysis

The chromatograms of most extracts were best eluted by the medium polar (CEF) and polar extractants (EMW) separating compounds in extracts into bands based on polarities. For the qualitative evaluation of a given substance, the *Rf* value on TLC is used as the parameter for comparison. The TLC fingerprints of the different chromatograms with the various solvent systems are shown in Figures 2, 3, and 4. According to the TLC evaluation scheme of Wagner and colleagues [24], the TLC fingerprint revealed the presence of flavonoids and triterpenoids.

Minimum inhibitory concentration

The MIC values of the plants extracts ranged from 0.078 mg/ml to 2.5 mg/ml after 24 h of incubation (Table 2). The average MIC values vary for the different bacterial species with the lowest observed in *S.enterica* serovar Typhi. Within 2 h incubation period, the average MIC of *E.autumnalis* and *P.guajava* against ESBL-positive serovar Typhimurium were 0.156 mg/ml and 0.078 mg/ml respectively. These values were comparable to that of the control gentamicin for the same pathogen as highlighted in Table 2. However, after 24 h incubation, the extract with most promising MIC value (0.078 mg/ml) was that of *A.arborescens*.
Table 2. MIC values (mg/ml) of plant extracts per organism compared with Gentamicin

<table>
<thead>
<tr>
<th>Bacteria codes</th>
<th>Plant extract codes</th>
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</thead>
<tbody>
<tr>
<td>EC 2H</td>
<td>AC: 1.25</td>
</tr>
<tr>
<td>EC 24H</td>
<td>0.625</td>
</tr>
<tr>
<td>EF 2H</td>
<td>0.625</td>
</tr>
<tr>
<td>EF 24H</td>
<td>0.625</td>
</tr>
<tr>
<td>PA 2H</td>
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<tr>
<td>PA 24H</td>
<td>0.125</td>
</tr>
<tr>
<td>SA 2H</td>
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<tr>
<td>SA 24H</td>
<td>0.312</td>
</tr>
<tr>
<td>STE- 2H</td>
<td>1.25</td>
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<tr>
<td>STE- 24H</td>
<td>0.312</td>
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<tr>
<td>STE+ 2H</td>
<td>1.25</td>
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<tr>
<td>STE+ 24H</td>
<td>0.125</td>
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<tr>
<td>SHF 2H</td>
<td>0.312</td>
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<tr>
<td>SHF 24H</td>
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<tr>
<td>SHS 2H</td>
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<tr>
<td>SHS 24H</td>
<td>0.625</td>
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Bacterial codes: EC 2H, E. coli MIC at 2h; EC 24H, E. coli MIC at 24h; EF 2H, Enterococcus faecalis MIC at 2h; EF 24H, E. faecalis MIC at 24h; PA 2H, Pseudomonas aeruginosa MIC at 2h; PA 24H, P. aeruginosa MIC at 24h; SA 2H, Staphylococcus aureus MIC at 2h; SA 24H, S. aureus MIC at 24h; SHF 2H, Shigella flexneri MIC at 2h; SHF 24H, S. flexneri MIC at 24h; SHS 2H, Shigella sonnei MIC at 2h; SHS 24H, S. sonnei MIC at 24h; STE- 2H, Salmonella Typhimurium ESBL negative MIC at 2h; STE- 24H, S. Typhimurium ESBL negative MIC at 24h; STE+ 2H, S. Typhimurium ESBL positive MIC at 2h; STE+ 24H, S. Typhimurium ESBL positive MIC at 24h.

Plant codes: AC, extract of Acacia mearnsii; AA, extract of Aloe arborescens; AS, extract of Aloe striata; HY, extract of Hypoxis latifolia; CU, extract of Cyathula uncinulata; MB, extract of Hernbastaedtia odorata; SC, extract of Scilla nervosa; PE, extract of Pelargonium sidoides; PS, extract of Psidium guajava; UM, extract of Hydnora comosa; E1, extract of Eucomis autumnalis; E2, extract of Eucomis comosa.

Discussion

Infectious enteric diseases including Salmonella are known to be particularly prevalent in the developing countries [25]. Salmonella enterica serovars have a broad host range however some Salmonella serovars are host-specific [26]. In this study, there was a striking predominance of infections with S. enterica serovar Typhi followed by S. enterica serovar Typhimurium. These two species as well as serovar Choleraesuis have been implicated worldwide as exclusive to human infections [27]. Most of the Salmonella strains were resistant to commonly used antibiotics. Reduced sensitivity to ciprofloxacin is an indication that more paediatric therapy options are being compromised. Antimicrobial resistance in S. enterica serotype Typhi was observed to be visibly increasing. Antibiotic resistance among disease causing Salmonella strains has compromised effectiveness of therapy [28] causing a drawback in the development of reliable therapies [29].

The majority of the Salmonella spp. showed pentavalent resistance to commonly used drugs. Over the years, there have been reports of a dramatic increase in multidrug resistant (MDR) salmonellae [30, 31]. In recent years, it is worrisome to note that the trend is still increasing [32] despite advances in technology. Multiple drug resistance enteric pathogens are a leading cause of morbidity and mortality worldwide [29, 33], bringing about increased hospitalization and cost of health care [34]. Of growing concern is the increasing trend in strains exhibiting ESBL production. There was a gradual increase in percent of isolates producing ESBLs from 16.2% to 37.8% over the 4 year period of study. Kruger et al [35], reported extensive ESBL production in strains of non typhoidal Salmonella in South Africa. In sub-Saharan Africa, factors such as poverty, malnutrition and increasing prevalence of HIV infected individuals are not helping matters [36].

The remarkable increase in drug-resistant microbes, coupled with the gap in the development of new antibiotics has called for continued search for alternative therapies to combat infectious diseases. To this end, of the crude extracts of medicinal plants screened against the selected enteric pathogens, Aloe arborescens, E. autumnalis and P. guajava showed promising antibacterial activity.
activities in comparison with gentamicin in terms of the MIC (Table 2). A previous study has reported the antibacterial activity of homoisoflavonoids isolated from E. comosa and E. humilis Bak. against Staphylococcus aureus [37]. Although activities of plant extracts are often more pronounced against Gram-positive pathogens [38], some extracts of plants in this study were not only active against Gram-positive but also against the selected Gram-negative isolates and in particular ESBL-positive strain of Salmonella. Aloe species are known to be widely used in ethnomedicine. According to Grace et al [39], most frequently cited medicinal uses of Aloe spp. were the treatment of infections and internal parasites, digestive ailments and injuries. The submission of Fabry et al [40] is that extract with MIC below 8 mg/ml are considered to have antimicrobial activities and since the MIC of most extract were below this value, it can be concluded that the crude extracts in this study had substantial activity against the selected enteric pathogens. In addition, it has been suggested that natural products with MIC values below 1 mg/ml are considered remarkable [41]. It is noteworthy that the MIC of P. guajava (0.078 mg/ml) and A. arborescens (0.078 mg/ml) against ESBL-positive S. enterica serovar Typhimurium at 2 h and 24 h incubation period, respectively, were even lower compared to gentamicin against selected isolates. Ramalivhana et al [42] demonstrated similar antibacterial activities with selected medicinal plants against methicillin-resistance Staphylococcus spp. and ESBL-positive enteric bacteria. ESBL-positive enteric pathogens have defied many of the commonly used antibiotics since beta-lactam drugs are among the most commonly prescribed drugs [43] and resistance to beta-lactam drugs confers resistance to third-generation and fourth generation cephalosporins and monobactams, and is frequently associated with co-resistance to fluoroquinolones, cotrimoxazole, tetracyclines, and aminoglycosides [44], hence limiting choice of therapeutic drugs. Thus these bioactive plants may serve as alternative therapy in the treatment of enteric diseases and may help alleviate the problem of drug resistance.

The considerable antibacterial activity of the crude extracts of A. arborescens and P. guajava against isolates exhibiting ESBL phenotypes among others may offer a means of mitigating the menace of multidrug resistant in these pathogens. This preliminary screening showed that some of the herbs used in traditional medicine in our area of study have potentials as antibacterial agents thereby there use in ethnomedicine.

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