Intracellular and membrane-damaging activities of methyl gallate isolated from *Terminalia chebula* against multidrug-resistant *Shigella* spp.

Saurabh Acharyya,[1]† Prodipta Sarkar,[1]† Dhira R. Saha,[2]
Amarendra Patra,[3] T. Ramamurthy[2] and Prasanta K. Bag[1]

Correspondence
Prasanta K. Bag
pkbbioc@caluniv.ac.in

[1]Department of Biochemistry, University of Calcutta, 35 Ballygunge, Circular Road, Kolkata 700019, India

[2]National Institute of Cholera and Enteric Diseases, P-33 CIT Road, Scheme-XM, Beliaghata, Kolkata 700010, India

[3]Department of Chemistry, University of Calcutta, 92 Acharya Prafulla Chandra Road, Kolkata 700009, India

Shigella spp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*) cause bacillary dysentery (shigellosis), which is characterized by bloody mucous diarrhoea. Although a variety of antibiotics have been effective for treatment of shigellosis, options are becoming limited due to globally emerging drug resistance. In the present study, *in vitro* antibacterial activity of methyl gallate (MG) isolated from *Terminalia chebula* was determined by performing MIC, minimal bactericidal concentration (MBC) and time-kill kinetic studies. Bacterial membrane-damaging activity of MG was determined by membrane perturbation and transmission electron microscopy (TEM). Cellular drug accumulation, cell infection and assessment of intracellular activities of MG and reference antibiotics were performed using HeLa cell cultures. The bactericidal activity of MG against multidrug-resistant (MDR) *Shigella* spp. in comparison with other commonly used drugs including fluoroquinolone was demonstrated here. TEM findings in the present study revealed that MG caused the total disintegration of inner and outer membranes, and leakage of the cytoplasmic contents of *S. dysenteriae*. The level of accumulation of MG and tetracycline in HeLa cells incubated for 24 h was relatively higher than that of ciprofloxacin and nalidixic acid (ratio of intracellular concentration/extracellular concentration of antibiotic for MG and tetracycline>ciprofloxacin and nalidixic acid). The viable number of intracellular *S. dysenteriae* was decreased in a time-dependent manner in the presence of MG (4×MBC) and reduced to zero within 20 h. The significant intracellular activities of MG suggested that it could potentially be used as an effective antibacterial agent for the treatment of severe infections caused by MDR *Shigella* spp.

INTRODUCTION

Shigella spp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*) cause bacillary dysentery (shigellosis), an invasive disease of the human colon which

reflects the capacity of these species to invade intestinal epithelial cells (Sansonetti, 2006). Invasion includes entry of the bacterium into epithelial cells, intracellular growth, intracellular movement and cell-to-cell spread, and eventually death of the host cell (Sansonetti, 2006). Shigellosis is a serious human health problem in both developing and industrialized countries. About 125 million cases of *Shigella* infection occur annually in Asia, of which ~14 000 are fatal (Bardhan *et al.*, 2010; Ghosh *et al.*, 2011). In the USA, there are up to 280 000 cases of shigellosis per year, most of which occur in preschool-age children with relatively mild disease (Bradley & Jackson, 2011). An increasing rate of ampicillin and trime-

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†These authors contributed equally to this paper.

Abbreviations: MDR, multidrug-resistant; MG, methyl gallate; MBC, minimal bactericidal concentration; PI, propidium iodide; TEM, transmission electron microscopy; TMP/SMX, trimethoprim/sulfamethoxazole.

One supplementary table is available with the online Supplementary Material.

thoprim/sulfamethoxazole (TMP/SMX) resistance has been reported, and multidrug-resistant (MDR) strains are becoming common. In an outbreak of MDR *S. sonnei* infection in the USA in 2005, 89 % of the strains were resistant to both ampicillin and TMP/SMX (CDC, 2006).

The distribution of serogroups of *Shigella* spp. differs from country to country: *S. dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydii* serogroups are predominant in developing countries, whilst *S. sonnei* is frequently reported in industrialized countries (Ghosh *et al.*, 2011). Effective antimicrobial therapy for shigellosis shortens the duration of clinical symptoms, reduces the severity of the dysentery and the threat of potential lethal complications, and prevents the spread of disease (WHO, 2005a; Nagano *et al.*, 2009). However, experimental treatment with first-line antimicrobial agents, including TMP/SMX, chloramphenicol, ampicillin, nalidixic acid and tetracycline, has become less effective due to the high prevalence of MDR clinical isolates amongst *Shigella* spp. (Dutta *et al.*, 2003; Pazhani *et al.*, 2005; Sivapalasingam *et al.*, 2006; von Seidlein *et al.*, 2006; Nagano *et al.*, 2009). Fluoroquinolones, ceftriaxone and pivmecillinam are the drugs of choice at present recommended by the World Health Organization for the treatment of MDR *Shigella* infection in adults, children and immunocompromised hosts, as these drugs are effective at reducing mortality caused by *Shigella* spp. (Nagano *et al.*, 2009; Traa *et al.*, 2010; Ghosh *et al.*, 2011). However, reduced susceptibility of *Shigella* spp., especially *S. dysenteriae* serotype 1, to fluoroquinolones has been reported (Sarkar *et al.*, 2003). In addition, the use of antimicrobials for the treatment of shigellosis varies from country to country (Ghosh *et al.*, 2011). Although pivmecillinam is being used in Bangladesh, it is not the drug of choice for the treatment of diarrhoea in many countries (Rahman *et al.*, 2007). Ceftriaxone is an ideal drug for the treatment of shigellosis in regions where fluoroquinolone resistance is common. However, isolation of ceftriaxone-resistant *Shigella* spp. has also been reported (Rahman *et al.*, 2007). The present antimicrobial resistance towards fluoroquinolones and ceftriaxone (Pazhani *et al.*, 2005; Rahman *et al.*, 2007; Wong *et al.*, 2010; Ghosh *et al.*, 2011) indicates that these drugs will no longer be useful, and emphasizes the need for using substitute drugs for the treatment of shigellosis.

Natural products have been used for centuries as traditional remedies to combat a variety of ailments, including infectious diseases (Ríos & Recio, 2005). Many plants conveniently available in India are used in traditional folklore medicine for the treatment of gastrointestinal disorders, such as cholera, diarrhoea and dysentery (Kala *et al.*, 2004). Many modern and novel drugs have their origin in traditional medicine and ethnopharmacology. *Terminalia chebula* Retz. (Combretaceae), commonly known as *haritaki*, is used for the treatment of diarrhoea, indigestion, etc., in traditional medicare systems (Kala *et al.*, 2004). We have demonstrated previously that *T. chebula* shows promising broad-spectrum antibacterial properties, inhibiting the strains of enteropathogenic bacteria (Acharyya

et al., 2009). Previously, *in vitro* activity of methyl gallate (MG) isolated from *Galla rhois* alone and in combination with reference antibiotics such as ciprofloxacin against *Salmonella*, *Escherichia*, *Clostridium* and *Enterobacter* spp. was studied (Choi *et al.*, 2009). However, the intracellular activity of MG against intracellular enteropathogens such as *Shigella* spp., the causative agents of shigellosis, has not yet been studied. In the present study, we demonstrated the intracellular and membrane-damaging activities of MG isolated from *T. chebula* against MDR *Shigella* spp.

METHODS

Plant material. MG was isolated, purified and identified from fruits of *T. chebula* as described previously by us (Acharyya *et al.*, 2012).

Bacterial strains. The clinical strains of *Shigella* spp. used were *S. flexneri* 2a (B294), *S. dysenteriae* 1 (NT4907), *S. boydii* 4 (BCH612) and *S. sonnei* (IDH00968SS) (strains were kindly provided by Dr Hemanta Koley, NICED, Kolkata, India). All the strains of *Shigella* spp. were positive for the Sereny test, and *ipaH* and *virF* genes. The strains were stored in 15 % glycerol stock at −70 °C.

Antibiotic susceptibility test. Antimicrobial susceptibility testing was performed by the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010) with commercially available disks (BD) of ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg) and tetracycline (30 μg) on Mueller–Hinton agar (BD) using *Escherichia coli* ATCC 25922 for quality control.

Antibacterial activity. The MICs and minimal bactericidal concentrations (MBCs) of MG and reference antibiotics (streptomycin, ciprofloxacin, ampicillin, tetracycline and nalidixic acid) were determined using broth microdilution as described previously (Thakurta *et al.*, 2007) following the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999). A final inoculum of $\sim 1 \times 10^6$ c.f.u. ml^{-1} was used and the concentrations of MG tested were 1–1000 μg ml^{-1}.

Selection of resistant mutants *in vitro*. The strain *S. dysenteriae* 1 was grown and maintained in Mueller–Hinton agar broth (MHB) in the presence of sublethal doses of MG (32 and 64 μg ml^{-1}) at 37 °C. Consecutive subcultures of *S. dysenteriae* 1 were exposed to sublethal doses of MG for 8 weeks and the bacteria were retested periodically (at 2 week intervals) to determine any increase in MIC following the method described above.

Time-kill kinetic study. Cultures of bacteria in MHB ($\sim 1 \times 10^6$ c.f.u. ml^{-1}) were incubated separately in the absence (control) and presence of drug at the MBC for 24 h at 37 °C. Samples of the bacterial cultures were removed at 2, 4, 8, 12 and 24 h to record survival counts, expressed as c.f.u. ml^{-1}. The surviving $\log_{10}$c.f.u. ml^{-1} was plotted against time.

Determination of mammalian cell toxicity. The haemolysis test was employed to determine cellular toxicity of MG (at concentrations of $1 \times$ to $4 \times$ MIC) as previously described (Situ & Bobek, 2000) using human red blood cells suspended in PBS (10 mM PBS, pH 7.4). The 100 % haemolytic control was the buffer containing 1 % Triton X-100 and the release of haemoglobin was determined at 540 nm. Toxicity of test compound was also assayed on HeLa cells as described previously (De La Fuente *et al.*, 2006).

Membrane perturbation assay. The membrane perturbation assay was performed using propidium iodide (PI) fluorescence detection as described previously (Tsao *et al.*, 2002). Bacteria (2×10^7 c.f.u. ml^{-1}) collected from the exponential phase were cultured with MG ($1 \times$ and $4 \times$ MBC) at 37 °C. At various intervals, the bacteria were collected and stained with PI (10 µg ml^{-1}) for 10 min, and after washing the cells, dye penetration was measured by the presence of fluorescence (excitation at 535 nm and emission at 560 nm).

Transmission electron microscopy (TEM) examination. Cultures of bacteria in MHB ($\sim 1 \times 10^7$ c.f.u. ml^{-1}) were incubated separately in the absence (control) and presence of drug at $1 \times$ MBC (256 µg ml^{-1}) for a period of 12 h at 37 °C. Samples of the bacterial cultures were removed at different time intervals and harvested (5000 *g*, 10 min, 4 °C). Cells were washed with PBS, prefixed in 3 % glutaraldehyde overnight at 4 °C and post-fixed in 1 % osmium tetroxide for 1 h at room temperature. The cells were dehydrated in a graded series of ethanol, cleared in propylene oxide and embedded in epoxy resin. Ultrathin sections were cut in a Leica Ultracut UCT ultramicrotome. After staining the sections with uranyl acetate and lead citrate, thin sections were examined using a FEI Tecnai 12 Biotwin transmission electron microscope.

Determination of cellular antibiotic accumulation. HeLa cells were cultured in RPMI 1640 medium supplemented with 10 % FCS in an atmosphere of 95 % air/5 % CO$_2$ as described previously (Bag *et al.*, 2008). Accumulation studies were performed as described previously (Carryn *et al.*, 2002; Barcia-Macay *et al.*, 2006). Cells were incubated with antibiotics for 24 h. Antibiotic concentration was measured by microbiological assay (by agar diffusion method) using *Bacillus subtilis* ATCC 6623 as the test organism.

Cell infection and assessment of intracellular activities of antibiotics. Invasion of HeLa cell monolayers by *Shigella* spp. has been used as a model which correlates with virulence in this organism, as penetration into epithelial cells is a requisite for virulence (Headley & Payne, 1990). In the present study, we used HeLa cells as a model to study *Shigella* infection and killing of intracellular bacteria. All experiments were performed with the HeLa cell line. The cells were maintained as described earlier. The cells (5×10^5 cells ml^{-1}) were infected by using a fresh inoculum of *S. dysenteriae* (2.5×10^6 c.f.u. ml^{-1}) that had been incubated for 1 h at 37 °C and washed extensively to remove non-firmly adherent bacteria (four successive sedimentations at 1300 r.p.m. followed by gentle resuspension in prewarmed sterile PBS), yielding a mean infection index of one bacterium per four HeLa cells (as determined by counting the c.f.u.) (Carryn *et al.*, 2002; Barcia-Macay *et al.*, 2006). The cells were then

incubated in fresh medium (with or without antibiotic) for up to 24 h. To ensure the absence of extracellular bacteria, the culture medium that contained cells with firmly adherent *S. dysenteriae* and that had not been exposed to antibiotics after the washing procedure described here were incubated at 37 °C for 48 h; no bacterial growth was detected. After 24 h incubation, the cells were collected by centrifugation, washed with ice-cold sterile PBS and lysed in distilled water. The lysates were then plated on nutrient agar at appropriate dilutions for determination of the number of viable bacteria by counting of the colonies (determination of the number of c.f.u.) and used for total cell protein measurement (Carryn *et al.*, 2002). All results were expressed as c.f.u. (mg HeLa cell protein)$^{-1}$.

RESULTS

The strains of *Shigella* spp. included in the present study were MDR clinical isolates. These were resistant/reduced-susceptible to chloramphenicol, furazolidone, norfloxacin, nalidixic acid, co-trimoxazole and tetracycline, and some of these isolates were resistant/reduced-susceptible to ciprofloxacin (fluoroquinolone), ampicillin and streptomycin (Table S1, available in the online Supplementary Material).

Mammalian cell toxicity

MG did not show cytotoxicity to human erythrocytes or HeLa cells (after 24 h of incubation) at concentrations of up to $4 \times$ MBC (results not shown).

MICs and MBCs of MG against *Shigella* spp

MG exhibited an MIC range of 128–256 µg ml^{-1} against *Shigella* spp., whereas the MBC was found to be in the range 256–512 µg ml^{-1} (Table 1). MICs and MBCs of streptomycin, tetracycline, ampicillin, nalidixic acid, ciprofloxacin and MG against *Shigella* spp. were determined in MHB and HeLa cell culture medium (RPMI 1640). Results are shown in Table 1.

Time-kill studies

The MBCs (*S. flexneri* 2a, 512 µg ml^{-1}; *S. dysenteriae* 1, 256 µg ml^{-1}; *S. boydii* 4, 256 µg ml^{-1}; and *S. sonnei*, 512

Table 1. MICs and MBCs (both µg ml^{-1}) of reference antibiotics and MG against *Shigella* spp.

ND, Not determined.

Test compound	*S. dysenteriae* 1 (NT4907)				*S. flexneri* 2a (B294)				*S. boydii* 4 (BCH612)				*S. sonnei* (IDH00968SS)			
	MHB		RPMI 1640		MHB		RPMI 1640		MHB		RPMI 1640		MHB		RPMI 1640	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Streptomycin	256	512	64	128	128	256	128	128	64	64	16	16	16	32	16	16
Ciprofloxacin	32	128	32	128	32	256	32	128	4	4	8	8	32	64	16	64
Ampicillin	256	256	ND	ND	256	256	ND	ND	4	4	ND	ND	8	8	ND	ND
Tetracycline	32	128	32	64	32	64	32	32	64	128	32	64	4	16	8	8
Nalidixic acid	256	512	256	512	128	128	64	256	8	32	16	16	512	512	256	256
MG	128	256	64	256	128	512	ND	ND	128	256	ND	ND	256	512	ND	ND

µg ml^{-1}) of MG showed a ∼6 log reduction in viable cell number (c.f.u.) within 20 h, compared with the untreated control (Fig. 1). At the MBC, MG showed a 4.74, 4.75, 3.68 and 4.13 log reduction in c.f.u. for *S. dysenteriae* 1, *S. boydii* 4, *S. flexneri* 2a and *S. sonnei* within 12 h, respectively, whereas tetracycline showed a 5.15, 4.92, 4.96 and 5.35 log reduction in c.f.u. for *S. dysenteriae* 1, *S. boydii* 4, *S. flexneri* 2a and *S. sonnei* within 12 h, respectively.

Selection of resistant mutants *in vitro*

After consecutive subculturing of *S. dysenteriae* 1 in the presence of sublethal doses of MG (32 and 64 µg ml^{-1}) for a period of 8 weeks, the bacteria did not show any increase in MIC and the MIC remained constant (i.e. 128 µg ml^{-1}).

TEM observations

Untreated cells of *S. dysenteriae* in MHB medium after 12 h of incubation at 37 °C showed a normal cell shape with an undamaged structure of the inner membrane, and the outer membrane layer was smooth and intact with slight folds (Fig. 2a). The periplasmic space was thin with a uniform appearance. However, when cells were grown in MHB supplemented with MG (256 µg ml^{-1}; 1 × MBC), significant membrane damage and intracellular structure changes were seen (Fig. 2b, c); both the outer membrane structure and the intracellular structure were fatally damaged. The membranes of the bacterial cells were distorted and intracellular structures were disorganized. Many empty cells were found in bacterial samples treated with MG for 12 h (Fig. 2c), indicating that the intracellular contents had
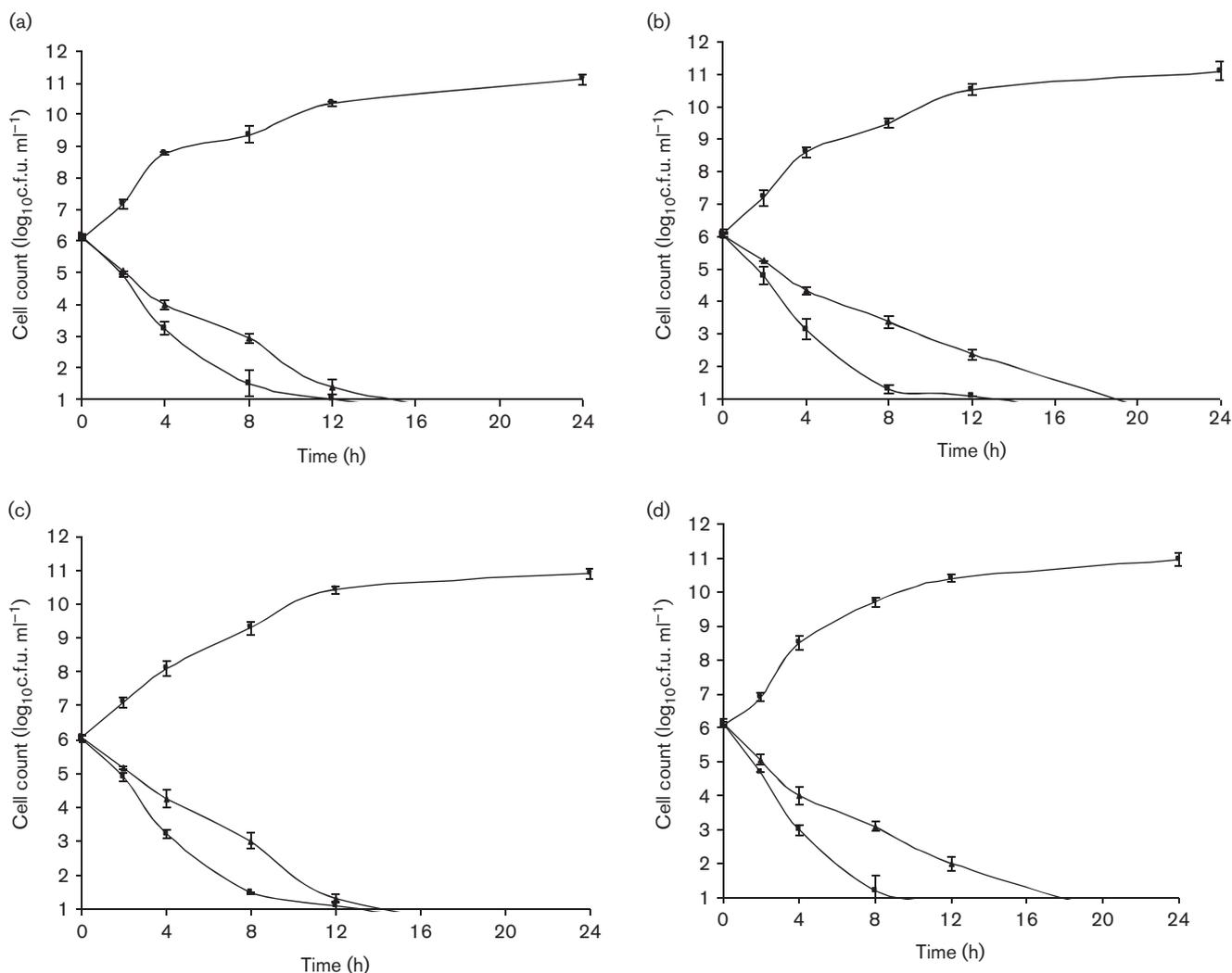

Fig. 1. Time-kill kinetic study of (a) *S. dysenteriae* 1, (b) *S. flexneri* 2a, (c) *S. boydii* 4 and (d) *S. sonnei* in the presence of MG at the MBC. Cells grown in the absence (control; ●), and in the presence of tetracycline (■) and MG (▲) at the MBC.
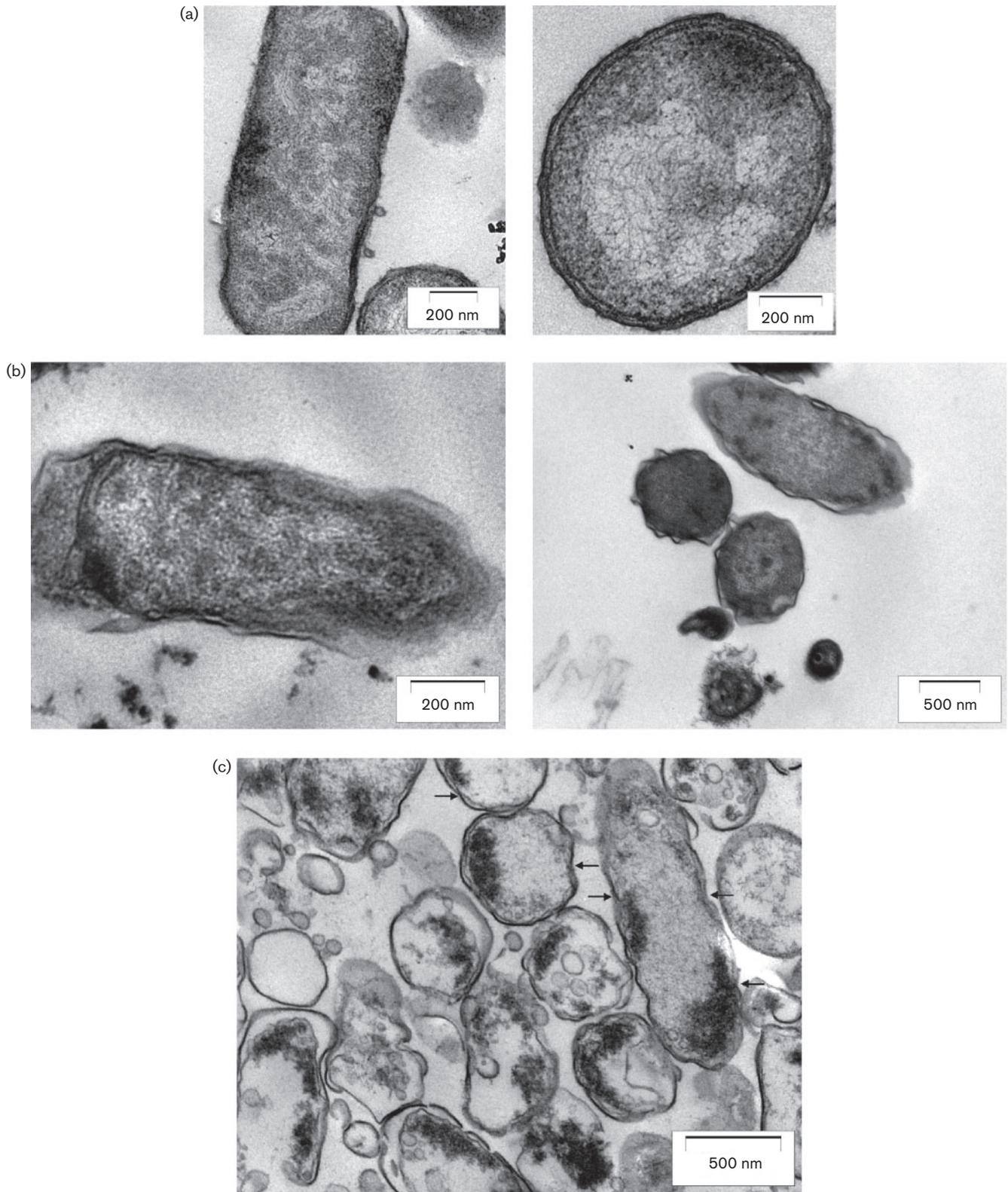
Fig. 2. (a) TEM images of *S. dysenteriae* 1 cells grown in antibiotic-free MHB medium (control) for 12 h. (b, c) TEM images of *S. dysenteriae* 1 cells grown in MHB supplemented with MG at 256 µg ml^{-1} (1×MBC) after incubation for 8 (b) and 12 h (c). Arrows indicate the leakage of intracellular contents and disorganization of cell membranes.
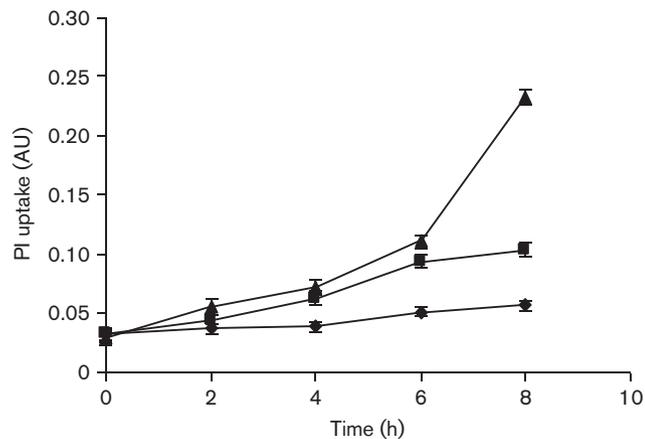
Fig. 3. Membrane perturbation assay by PI uptake after treatment with MG. At various time intervals, the bacterial cells were harvested and stained with PI. PI uptake was expressed as the absorbance in atomic units (AU; mean ± SD). Cells grown in the absence of MG (●); cells grown in presence of 1 × (■) and 4 × (▲) MBC of MG.

leaked out of the cells due to damage to the cell membrane. In addition, in the membrane perturbation assay, it was found that PI uptake increased with time after treatment with MG in a dose-dependent manner (Fig. 3).

Cellular antibiotic accumulation and assessment of intracellular activities of antibiotics

Table 2 shows the levels of accumulation of ciprofloxacin, tetracycline, nalidixic acid and MG in HeLa cells incubated for 24 h in the presence of the drugs at 1 × MBC. The level of accumulation of MG and tetracycline was relatively higher than that of ciprofloxacin and nalidixic acid, as the ratio of intracellular concentration/extracellular concentration of antibiotic (C_c/C_e) was relatively higher for MG and tetracycline than that of ciprofloxacin and nalidixic acid.

Intracellular activity of MG and other reference antibiotics at 4 × MBC is shown in Fig. 4. At 1 h post-infection, reference antibiotic or MG was added to *S. dysenteriae*-infected HeLa cells and viability of intracellular bacteria was measured at different time points (0, 2, 5 and 24 h) of incubation. The viable number of intracellular bacteria was decreased in a time-dependent manner in the presence of the drugs. MG at 4 × MBC reduced the cell number [reduction of ~4.5 $\log_{10}$c.f.u. (mg HeLa cells protein)$^{-1}$] of intracellular *S. dysenteriae* to zero (below the detection level) within 24 h at 37 °C. These observations confirmed the intracellular activity of MG.

DISCUSSION

Shigella is a highly infectious pathogenic micro-organism and as few as 10–100 bacterial cells can cause the disease

Table 2. Cellular accumulation of antibiotics in HeLa cells after 24 h incubation

MBCs of nalidixic acid, ciprofloxacin, tetracycline and MG were 512, 128, 64 and 256 μg ml^{-1}, respectively, against *S. dysenteriae*. Antibiotic concentration was determined by agar diffusion assay against *B. subtilis*.

Antibiotic	Antibiotic added (μg ml^{-1})	C_c/C_e*
Control	0	0
Tetracycline	64	39.63 ± 0.10
Ciprofloxacin	128	21.20 ± 2.12
Nalidixic acid	512	11.21 ± 0.70
MG	256	32.74 ± 2.12

*C_c/C_e ratio (mean ± SD, n=5) was calculated from the drug contents per mg of cell protein by using a conversion factor of 5 μl (mg cell protein)$^{-1}$.
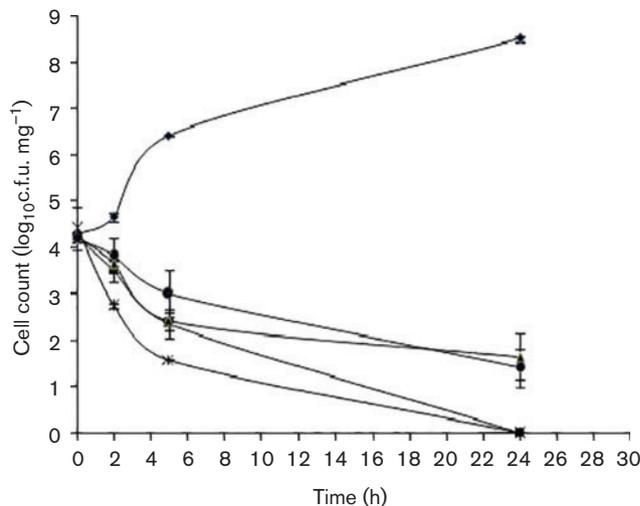

Fig. 4. Influence of time on the rate and extent of the activities of the antibiotics against intracellular *S. dysenteriae* 1 upon incubation at a fixed extracellular concentration (4 × MBC). The graphs show the change in c.f.u. from time 0 ($\log_{10}$c.f.u.; mean ± SD; n=3) in HeLa cells (intracellular) per mg of cell protein. Intracellular growth in the absence (control; ◆) of antibiotic; intracellular growth in the presence of MG (■), nalidixic acid (▲), tetracycline (●) and ciprofloxacin (*).

in adults (Kweon, 2008). The choice of drugs for the treatment of shigellosis has become limited due to the emergence of MDR strains of *Shigella* spp. (Rahman *et al.*, 2007; Ghosh *et al.*, 2011). It was found in the present study that MG was significantly active against the MDR (including fluoroquinolone-resistant) clinical isolates of *Shigella* spp. MG at 1 × MBC exhibited a time-dependent killing effect against *S. flexneri*, *S. dysenteriae*, *S. boydii* and *S. sonnei*, and could reduce the number of viable cells (c.f.u. count) of these

species below the detection limit within 20 h. Amongst four species and many subtypes of *Shigella*, *S. dysenteriae* 1 is the only *Shigella* that causes epidemics and pandemics, distinguished by high fever, cramps, seizures, bloody diarrhoea and dysentery, and haemolytic uraemic syndrome, and shows enormously high fatality rates amongst children and adults (Sur *et al.*, 2004; Pozsgay *et al.*, 2007; McKenzie *et al.*, 2008; Barman *et al.*, 2011). *S. dysenteriae* 1 causes recurrent disease in Asian countries such as India and Bangladesh (Dutta *et al.*, 2003; von Seidlein *et al.*, 2006; Emch *et al.*, 2008). Several outbreaks of *S. dysenteriae* 1 infection were associated with fluoroquinolone-resistant bacteria (Sarkar *et al.*, 2003; WHO, 2005b). In addition, amongst the strains of *Shigella* studied here, *S. dysenteriae* 1 showed the highest overall resistance in terms of MICs and MBCs against all of the reference drugs tested here, including quinolones and fluoroquinolones. Hence, *S. dysenteriae* 1 was chosen for further studies.

TEM findings in the present study revealed that exposure of *S. dysenteriae* 1 to MG at $1 \times$ MBC resulted in disintegration of the bacterial cell membrane. Our TEM study demonstrated that MG caused damage to the outer membrane of *Shigella*, total disintegration of the inner and outer membranes, and leakage of the cytoplasmic contents. The increased uptake of PI in the MG-treated cells of *S. dysenteriae* in our study further confirmed that MG altered the cell membrane structure, resulting in disruption of the permeability barrier of the microbial membrane structures. This antibacterial effect of MG observed in this study is consistent with the results reported recently (Sánchez *et al.*, 2013) where the membrane-damaging activity of MG isolated from *Acacia farnesiana* against *Vibrio cholerae* was demonstrated. In addition, bactericidal activity of antibiotics and natural products such as paenibacterin, curcumin, etc., has been demonstrated previously, which was associated with disruption of the outer membrane of Gram-negative bacteria/damage of the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria (Huang & Yousef, 2014; Tyagi *et al.*, 2015). The bacterial cytoplasmic membrane is indispensable to bacterial life as it contains one-third of the proteins in a cell and is the location for many crucial processes (Hurdle *et al.*, 2011; Huang & Yousef, 2014). Therefore, these antibacterial agents, including MG, may interfere with numerous cellular functions by damaging the cell membrane (Hurdle *et al.*, 2011; Huang & Yousef, 2014).

Various antibiotics have been effective for the treatment of shigellosis, although options are becoming limited due to globally emerging drug resistance (Sack *et al.*, 2001). Originally, both sulfonamides and tetracycline were effective, but *Shigella* strains rapidly developed resistance to these agents (Sack *et al.*, 2001). Ampicillin and TMP/SMX were then used, and continue to be effective in many industrialized countries. Unfortunately, in many parts of the world, strains of all species of *Shigella* have become resistant to these low-cost agents and neither can now be confidently used as empiric therapy for shigellosis (Sack *et al.*, 2001). One of the few remaining, relatively inexpensive

and effective drugs for shigellosis is the quinolone, nalidixic acid. Unfortunately, resistance to nalidixic acid is also common; in regions where it was introduced to treat epidemic shigellosis due to shiga bacillus, resistance developed within 6 months (Sack *et al.*, 2001). Although fluoroquinolones and ceftriaxone are able to penetrate mammalian cells and are effective against the MDR strains of *Shigella* spp., several reports on antimicrobial resistance/reduced susceptibility of *Shigella* spp., especially *S. dysenteriae*, towards fluoroquinolones and ceftriaxone indicate that these drugs will no longer be useful (Sarkar *et al.*, 2003; Rahman *et al.*, 2007; Nagano *et al.*, 2009; Traa *et al.*, 2010; Ghosh *et al.*, 2011). Moreover, these drugs are expensive. Thus, there are very few antibiotic options. In our study, after consecutive subculturing of *S. dysenteriae* 1 in the presence of sublethal doses of MG for 8 weeks, the bacteria did not show any increase in MIC, indicating that exposure to MG for this long period of time did not produce any MG resistance. It has been suggested previously that unlike other conventional antibiotics, membrane-active agents are less likely to mediate antibiotic resistance in treated bacteria (Hurdle *et al.*, 2011; Huang & Yousef, 2014). As MG clearly damages bacterial cytoplasmic membranes and *S. dysenteriae* treated with MG did not show any MG resistance, in terms of the challenges of antibacterial resistance, MG could be a potential candidate for the treatment of emerging infectious disease such as shigellosis. We have demonstrated here the killing effect of MG on extracellular and intracellular (in HeLa cells) growth of *S. dysenteriae*. Correlation of the killing effect of MG and tetracycline on *S. dysenteriae* between its extracellular and intracellular (in HeLa cells) growth has been compared by combining data for MG and tetracycline from Figs 1 and 4 (Fig. 5). It was interesting to note that there was no significant difference between the two sets of data ($P \geq 0.4659$) for MG and tetracycline. The correlation coefficients between the effect on extracellular and intracellular growth of MG and tetracycline are 0.9999 and 0.9973, respectively. In addition, we have demonstrated here the significant penetration and accumulation of MG inside HeLa cells. The C_c/C_e ratios for ciprofloxacin, tetracycline and MG were 21.20, 39.63 and 32.74, respectively, i.e. ciprofloxacin, tetracycline and MG were accumulated up to 21.20-, 39.63- and 32.74-fold inside HeLa cells, respectively. Hence, it can be suggested following the analysis of Barcia-Macay *et al.* (2006) that ciprofloxacin, MG and tetracycline achieved high levels (21.20- to 39.63-fold) of accumulation. Penetration of antibiotics in effective concentrations inside cells is one of the important criteria to kill intracellular pathogens (Kadurugamuwa & Beveridge, 1998). The intracellular killing activity of MG was comparable with that of ciprofloxacin, and stronger than that of nalidixic acid and tetracycline.

The most striking features of MG were its activity against clinical isolates of *Shigella*, which are resistant/reduced-susceptible to the antimicrobial agents recommended by the World Health Organization, including TMP/SMX,
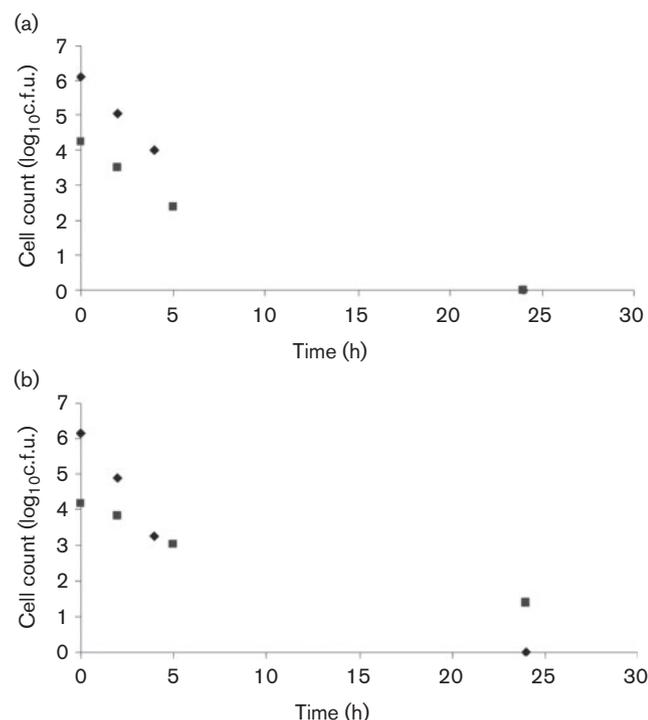
Fig. 5. Combined data for MG and tetracycline from Figs 1 and 4. The values on the y-axes indicate the $\log_{10}$c.f.u. (ml broth)$^{-1}$ (♦) or $\log_{10}$c.f.u. (mg HeLa cell protein)$^{-1}$ (■). Correlation of killing effect of (a) MG and (b) tetracycline on *S. dysenteriae* between extracellular (♦) and intracellular (HeLa cells) (■) growth. Statistical analysis (analysis of covariance) shows that there was no significant difference between the two sets of data ($P \geqslant 0.4659$) for MG and tetracycline. Correlation coefficients between the effect on extracellular and intracellular growth of MG and tetracycline are 0.9999 and 0.9973, respectively.

chloramphenicol, ampicillin, nalidixic acid, tetracycline and fluoroquinolone like ciprofloxacin, and its uniform bactericidal power against four species of MDR *Shigella*. In addition, exposure of *S. dysenteriae* to MG for a long period of time did not produce any MG resistance. Considering these results and the significant inhibitory, *in vitro* and intracellular bactericidal activities of MG demonstrated here, it is suggested that it could be considered as a potential effective antibacterial agent for the treatment of severe infections caused by MDR *Shigella* spp., particularly those resistant/reduced-susceptible to most of the recommended antibiotics. However, it is so far unclear whether the membrane damage was the sole bactericidal mode of MG action. It may have an effect on the cellular proteins and nucleic acid in addition to the membranes. The binding with the membrane and the transport of MG across the membrane and its effect on cell components could be studied in detail in the future.

REFERENCES

Acharyya, S., Patra, A. & Bag, P. K. (2009). Evaluation of the antimicrobial activity of some medicinal plants against enteric bacteria with particular reference to multi-drug resistant *Vibrio cholerae*. *Trop J Pharm Res* **8**, 231–237.

Acharyya, S., Patra, A. & Bag, P. K. (2012). A comparative study on antioxidant potential of nine Indian medicinal plants. *CIBTECH J Pharm Sci* **1**, 16–23.

Bag, P. K., Bhowmik, P., Hajra, T. K., Ramamurthy, T., Sarkar, P., Majumder, M., Chowdhury, G. & Das, S. C. (2008). Putative virulence traits and pathogenicity of *Vibrio cholerae* non-O1, non-O139 isolates from surface waters in Kolkata, India. *Appl Environ Microbiol* **74**, 5635–5644.

Barcia-Macay, M., Seral, C., Mingeot-Leclercq, M. P., Tulkens, P. M. & Van Bambeke, F. (2006). Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. *Antimicrob Agents Chemother* **50**, 841–851.

Bardhan, P., Faruque, A. S., Naheed, A. & Sack, D. A. (2010). Decrease in shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. *Emerg Infect Dis* **16**, 1718–1723.

Barman, S., Kumar, R., Chowdhury, G., Rani Saha, D., Wajima, T., Hamabata, T., Ramamurthy, T., Balakrish Nair, G., Takeda, Y. & Koley, H. (2011). Live non-invasive *Shigella dysenteriae* 1 strain induces homologous protective immunity in a guinea pig colitis model. *Microbiol Immunol* **55**, 683–693.

Bradley, J. S., Jackson, M. A. & Committee on Infectious Diseases (2011). The use of systemic and topical fluoroquinolones. *Pediatrics* **128**, e1034–e1045.

Carryn, S., Van Bambeke, F., Mingeot-Leclercq, M. P. & Tulkens, P. M. (2002). Comparative intracellular (THP-1 macrophage) and extracellular activities of β-lactams, azithromycin, gentamicin, and fluoroquinolones against *Listeria monocytogenes* at clinically relevant concentrations. *Antimicrob Agents Chemother* **46**, 2095–2103.

CDC (2006). Outbreaks of multidrug-resistant *Shigella sonnei* gastroenteritis associated with day care centers: Kansas Kentucky, Missouri, 2005. *MMWR Morb Mortal Wkly Rep* **55**, 1068–1071.

Choi, J.-G., Kang, O.-H., Lee, Y.-S., Oh, Y.-C., Chae, H.-S., Jang, H.-J., Shin, D.-W. & Kwon, D.-Y. (2009). Antibacterial activity of methyl gallate isolated from Galla Rhois or carvacrol combined with nalidixic acid against nalidixic acid resistant bacteria. *Molecules* **14**, 1773–1780.

CLSI (2010). *Performance Standards for Antimicrobial Susceptibility Testing; 18th Information Supplement M100-S20.* Wayne, PA: Clinical and Laboratory Standards Institute.

De La Fuente, R., Sonawane, N. D., Arumainayagam, D. & Verkman, A. S. (2006). Small molecules with antimicrobial activity against *E. coli* and *P. aeruginosa* identified by high-throughput screening. *Br J Pharmacol* **149**, 551–559.

Dutta, S., Dutta, S., Dutta, P., Matsushita, S., Bhattacharya, S. K. & Yoshida, S. (2003). *Shigella dysenteriae* serotype 1, Kolkata, India. *Emerg Infect Dis* 9, 1471–1474.

Emch, M., Ali, M. & Yunus, M. (2008). Risk areas and neighborhood-level risk factors for *Shigella dysenteriae* 1 and *Shigella flexneri*. *Health Place* 14, 96–105.

Ghosh, S., Pazhani, G. P., Chowdhury, G., Guin, S., Dutta, S., Rajendran, K., Bhattacharya, M. K., Takeda, Y., Niyogi, S. K. & other authors (2011). Genetic characteristics and changing antimicrobial resistance amongst *Shigella* spp. isolated from hospitalized diarrhoeal patients in Kolkata, India. *J Med Microbiol* 60, 1460–1466.

Headley, V. L. & Payne, S. M. (1990). Differential protein expression by *Shigella flexneri* in intracellular and extracellular environments. *Proc Natl Acad Sci U S A* 87, 4179–4183.

Huang, E. & Yousef, A. E. (2014). The lipopeptide antibiotic paenibacterin binds to the bacterial outer membrane and exerts bactericidal activity through cytoplasmic membrane damage. *Appl Environ Microbiol* 80, 2700–2704.

Hurdle, J. G., O'Neill, A. J., Chopra, I. & Lee, R. E. (2011). Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat Rev Microbiol* 9, 62–75.

Kadurugamuwa, J. L. & Beveridge, T. J. (1998). Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using *Shigella flexneri* membrane vesicles. *Antimicrob Agents Chemother* 42, 1476–1483.

Kala, C. P., Farooquee, N. A. & Dhar, U. (2004). Prioritization of medicinal plants on the basis of available knowledge, existing practices and use value status in Uttaranchal, India. *Biodivers Conserv* 13, 453–469.

Kweon, M. N. (2008). Shigellosis: the current status of vaccine development. *Curr Opin Infect Dis* 21, 313–318.

McKenzie, R., Venkatesan, M. M., Wolf, M. K., Islam, D., Grahek, S., Jones, A. M., Bloom, A., Taylor, D. N., Hale, T. L. & Bourgeois, A. L. (2008). Safety and immunogenicity of WRSd1, a live attenuated *Shigella dysenteriae* type 1 vaccine candidate. *Vaccine* 26, 3291–3296.

Nagano, Y., Nagano, N., Wachino, J., Ishikawa, K. & Arakawa, Y. (2009). Novel chimeric beta-lactamase CTX-M-64, a hybrid of CTX-M-15-like and CTX-M-14 beta-lactamases, found in a *Shigella sonnei* strain resistant to various oxyimino-cephalosporins, including ceftazidime. *Antimicrob Agents Chemother* 53, 69–74.

NCCLS (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*; Approved Standards M7-A4. Wayne, PA: National Committee for Clinical Laboratory Standards.

NCCLS (1999). *Methods for Determining Bactericidal Activity of Antimicrobial Agents*; Approved Guideline M26-A. Wayne, PA: National Committee for Clinical Laboratory Standards.

Pazhani, G. P., Ramamurthy, T., Mitra, U., Bhattacharya, S. K. & Niyogi, S. K. (2005). Species diversity and antimicrobial resistance of *Shigella* spp. isolated between 2001 and 2004 from hospitalized children with diarrhoea in Kolkata (Calcutta), India. *Epidemiol Infect* 133, 1089–1095.

Pozsgay, V., Kubler-Kielb, J., Schneerson, R. & Robbins, J. B. (2007). Effect of the nonreducing end of *Shigella dysenteriae* type 1 O-specific oligosaccharides on their immunogenicity as conjugates in mice. *Proc Natl Acad Sci U S A* 104, 14478–14482.

Rahman, M., Shoma, S., Rashid, H., El Arifeen, S., Baqui, A. H., Siddique, A. K., Nair, G. B. & Sack, D. A. (2007). Increasing spectrum in antimicrobial resistance of *Shigella* isolates in Bangladesh: resistance to azithromycin and ceftriaxone and decreased susceptibility to ciprofloxacin. *J Health Popul Nutr* 25, 158–167.

Ríos, J. L. & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *J Ethnopharmacol* 100, 80–84.

Sack, D. A., Lyke, C., McLaughlin, C. & Suwanvanichkij, V. (2001). *Antimicrobial Resistance in Shigellosis, Cholera and Campylobacteriosis*. Geneva: World Health Organization.

Sánchez, E., Heredia, N., Camacho-Corona, M. R. & García, S. (2013). Isolation, characterization and mode of antimicrobial action against *Vibrio cholerae* of methyl gallate isolated from *Acacia farnesiana*. *J Appl Microbiol* 115, 1307–1316.

Sansonetti, P. J. (2006). The bacterial weaponry: lessons from *Shigella*. *Ann N Y Acad Sci* 1072, 307–312.

Sarkar, K., Ghosh, S., Niyogi, S. K. & Bhattacharya, S. K. (2003). *Shigella dysenteriae* type 1 with reduced susceptibility to fluoroquinolones. *Lancet* 361, 785.

Situ, H. & Bobek, L. A. (2000). *In vitro* assessment of antifungal therapeutic potential of salivary histatin-5, two variants of histatin-5, and salivary mucin (MUC7) domain 1. *Antimicrob Agents Chemother* 44, 1485–1493.

Sivapalasingam, S., Nelson, J. M., Joyce, K., Hoekstra, M., Angulo, F. J. & Mintz, E. D. (2006). High prevalence of antimicrobial resistance amongst *Shigella* isolates in the United States tested by the National Antimicrobial Resistance Monitoring System from 1999 to 2002. *Antimicrob Agents Chemother* 50, 49–54.

Sur, D., Ramamurthy, T., Deen, J. & Bhattacharya, S. K. (2004). Shigellosis: challenges & management issues. *Indian J Med Res* 120, 454–462.

Thakurta, P., Bhowmik, P., Mukherjee, S., Hajra, T. K., Patra, A. & Bag, P. K. (2007). Antibacterial, antisecretory and antihemorrhagic activity of *Azadirachta indica* used to treat cholera and diarrhea in India. *J Ethnopharmacol* 111, 607–612.

Traa, B. S., Walker, C. L., Munos, M. & Black, R. E. (2010). Antibiotics for the treatment of dysentery in children. *Int J Epidemiol* 39 (Suppl 1), i70–i74.

Tsao, N., Luh, T.-Y., Chou, C.-K., Chang, T.-Y., Wu, J.-J., Liu, C.-C. & Lei, H.-Y. (2002). *In vitro* action of carboxyfullerene. *J Antimicrob Chemother* 49, 641–649.

Tyagi, P., Singh, M., Kumari, H., Kumari, A. & Mukhopadhyay, K. (2015). Bactericidal activity of curcumin I is associated with damaging of bacterial membrane. *PLoS One* 10, e0121313.

von Seidlein, L., Kim, D. R., Ali, M., Lee, H., Wang, X., Thiem, V. D., Canh, G., Chaicumpa, W., Agtini, M. D. & other authors (2006). A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med* 3, e353.

Wong, M. R., Reddy, V., Hanson, H., Johnson, K. M., Tsoi, B., Cokes, C., Gallagher, L., Lee, L., Plentsova, A. & other authors (2010). Antimicrobial resistance trends of Shigella serotypes in New York City, 2006-2009. *Microb Drug Resist* 16, 155–161.

WHO (2005a). Shigellosis disease burden, epidemiology and case management. *Wkly Epidemiol Rec* 80, 94–99.

WHO (2005b). *Guidelines for the Control of Shigellosis, Including Epidemics Due to Shigella dysenteriae 1*. Geneva: World Health Organization.