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Full Length Research Paper

Cyclic depsipeptide producing fluorescent pseudomonads exerts antifungal activity against fungal pathogens of maize *(Zea mays)*

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Cyclic lipopeptides (CLPs) with antibiotic, biosurfactant producing fluorescent pseudomonads were isolated from sugar beet-maize intercropped in sandy loam soils at Maize Research Station, Vagarai, TNAU. Approximately 20 fluorescent pseudomonads from sandy loam soils were isolated by using two different growth media. The strains were distinguished based on their growth, CFU/g, fluorescence, and pigment production. Growth inhibition of maize pathogens by CLP producing *fluorescent pseudomonads* strains were studied by dual culture experiments. The impact of CLP producing flurorescent pseudomonads strain on the zoospores of Downy mildew pathogen of maize was studied by direct microscopy and encysted zoospores were observed. *Invitro,* biochemical experiments confirmed the presence of Viscosinamide producing strain among the fluorescent pseudomonads isolates in terms of utilization of C and N sources. The particular strain was tested for its growth promoting activity by treating the maize seeds for their germination, and seedling vigour performance. Fluorescent pseudomonads can be affiliated to group under CLP producing biotypes/biovars. Purification of CLP (Viscosinamide) and characterization by HPLC analysis was carried out. Pot culture experiments were conducted to test the performance of CLP producing *Pf* strains in maize crop for testing their disease resistance. These biovars with antibiotic properties are the potential targets for the disease management in maize. CLPs in general receive considerable attention as potent antimicrobial drugs.

Key words: Cyclic lipopeptides, fluorescent pseudomonads, viscosinamide, zoospores, antifungal.

INTRODUCTION

Biosurfactants are found to be structurally diverse in nature and are commonly synthesized by micro-organisms. The

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structure of biosurfactants comprise of a hydrophilic moiety of amino acids or peptide, anionsor cations, mono-or polysacharides, and a hydrophobic moiety consisting of fatty acids. Biosurfactants have been commonly classified as: (i) Low molecular-weight molecules, which decrease surface tension efficiently; and (ii) High-molecular weight polymers which bind to surfaces (Rosenberg and Ron, 1997). Low-molecular weight biosurfactants belongs to the classes of glycolipids or lipopeptides. Basically, biosurfactants have a number of advantages over chemical surfactants such as lower toxicity, higher biodegradability, environmental conditions (for instance temperature, pH and salinity). Large group of microbes are capable of producing biosurfactants, which includes *Pseudomonas* spp. strains producing rhamnolipids (Lang and Wullbrandt, 1999; Providenti et al., 1995; Shreve et al., 1995) and *Bacillus* sp. strains, producing surfactins (Fuma et al., 1993; Yakimov et al., 1995). Within the group of biosurfactant producing microbes, fluorescent pseudomonads received more attention for the past two decades (Hotte and Altier, 2010; Raaijmakers and Mazzola, 2012; Olorunleke et al., 2015).

The role and applications of biosurfactants (mainly glycolipids and lipopeptides) have been investigated from medicinal and therapeutic properties. Cameotra and Makkar (2004) reviewed properties of biosurfactants as antimicrobial agents, immunoregulators, adhesives and desorptive agents in surgical procedures. Various Pseudomonas biocontrol strains produce CLP type biosurfactants (Olorunleke et al., 2015). CLPs are amphiphilic molecules composed of a cyclic oligopeptide lactone ring coupled to a fatty acid tail (Raaijmakers et al., 2010). CLPs possess broad spectrum of antibiosis against bacteria, fungi, protozoa and human tumor cell lines (Raaijmakers et al., 2010; Roongsawang et al., 2010). They are potential pharmaceutical candidates for the biological control of plant pathogens (Banat et al., 2010, Sachdev and Cameotra, 2013). Many cyclic lipopeptides are antimicrobial agents, among them Viscosinamide produced by *Pseudomonas* spp. isolated from sugarbeet rhizosphere has antibiotic properties towards root-pathogenic fungi (Nielsen et al., 2003). Screening of *Pseudomonas* spp. for their capability to produce cyclic lipopeptides is an important criterion for the selection of biological control agents, as it may be used as single strain/consortium of strains to improve multiple antagonistic traits.

MATERIALS AND METHODS

Isolation of surfactant producing *Pseudomonas* spp. strains

Soil samples were collected from loamy sand, where maize crop was intercropped with sugarbeet and kept at 5°C until use. The samples were weighed for 50 g in polythene vials with the bulk density 1.1 g cm^{-3}. Maize seeds were sown in vials (3 seeds/vial) and kept in 15°C under 16 h light and 8 h dark cycle. The seedlings were uprooted along with adhering soils and transferred to 10 ml sterile 0.9% Nacl. The sample was vortexed for 1 min and sonicated for 0.5 min and plated in solid media.

High density population of *Pseudomonas spp*. was obtained in two different media: (i) On King's B medium fluorescent *Pseudomonas* spp. were detected by exposing the agar plates with UV light (254 nm) and the fluorescent colonies were randomly picked. (ii) Gould's S1 medium, containing 10 g sucrose, 10 ml of glycerol, 5 g of casamino acids, 1 g of $NaHCO_3$, 1 g of $MgSO_4.7H_2O$, 2.3 g of K_2HPO_4, 1.2 g of sodium lauryl sulphate and 15 g of agar per liter was autoclaved, and then 5 ml of 100 mg of trimethoprim, 8.5 ml of methanol, and 16.5 ml of Milli-Q water was added to the medium. The colonies appearing in Gould´S1 selective medium were eligible for random picking.

Isolates from the two media were further streaked onto Gould' S1 agar and checked for fluorescence before culturing in 3 ml of Luria-Bertani medium per liter containing 10 g of tryptone, 5 g of yeast extract, 10 g of Nacl, and 1 g of glucose pH 7.2 for subsequent preservation at -80°C.

Swarming and biofilm assays

Bacterial cells grown for 24 h on GS1 (Gould's S1) medium agar plates were dissolved in sterile distilled water to a final density of 10^9 CFU ml^{-1} (OD_{600} = 1), pelleted by centrifugation and washed once with sterile distilled water. Swarming assays were performed on soft agar plates (KB medium with 0.6% (W/V) agar, five microlitres of the cell suspension were placed in the centre of a soft agar plate. The ability of the bacterial colony to spread was evaluated after 24, 48 and 72 h of incubation at 25°C (Neilson et al., 1999).

The biofilm assays were performed in flat-bottom non-detachable 96 wells plates (Nunc.Immuno^{TM}MicroWell^{TM}, SIGMA-ALDRICH, USA) according to the methods described by O'Toole et al. (1999) and Bruine de Bruin et al. (2007). The 96 wells were filled with 180 µl of Gould's S1 medium and 20 µl bacterial suspension (1×10^9 cells ml^{-1}) and 20 µl bacterial suspension (1×10^9 cells ml^{-1}) and incubated for 24 h at 25°C. Biofilms were stained with crystal violet and visualized at 600 nm (Bruine de Bruin et al., 2007). The biofilms were observed in side walls of the 96 well plates and the OD was measured at 600 nm.

Zoosporicidal and antifungal activity

Bacterial cell suspensions (10^9CFU ml^{-1}) were prepared from colonies grown on GS1 plates for 48 h at 25°C. A 10 µl aliquot of the bacterial cell suspension was mixed on a glass slide with downy mildew zoospores (10^4 ml^{-1}) in a 1:1 ratio (v:v). Zoospore lysis was observed microscopically at 100X magnification for up to 2 min. Dual culture inhibition assays were performed by spot inoculating *fluorescent Pseudomonas* to the edge of an agar plate and incubation for 3 days at 25°C followed by placing a fungal agar plug (5 mm diameter) to the centre of the plate and incubation at diverse temperatures for up to 14 days.

Growth analysis

Seeds were surface-sterilized for 5 min in 1% (w/v) sodium hypochlorite, rinsed in sterile distilled water, and allowed for uniform coating in talc formulations of *Pf* strains overnight at 25°C. Seeds were then sown on a layer of brown germination towel of thin, wet

Table 1. Growth, fluoresence, CFU and pigment production in two selective media after 24 h at 23°C

Pf strains*	Growth		CFU/g		Fluorescence		Pigment production	
	King's B	MGS1	King's B	MGS1	King's B	MGS1	King's B	MGS1
MSBR1	+	-	90	-	+	-	+	-
MSBR2	+	+	100	15	+	+	+	+
MSBR3	+	-	70	-	+	-	+	-
MSBR4	+	-	80	-	+	-	+	-
MSBR5	+	-	75	20	+	+	+	+
MSBR6	+	-	95	-	+	-	+	-
MSBR7	+	-	66	-	+	-	+	-
MSBR8	+	-	95	-	+	-	+	-
MSBR9	+	-	63	-	+	-	+	-
MSBR10	+	-	70	25	+	+	+	+
MSBR11	+	-	85	-	+	-	+	-
MSBR12	+	+	100	30	+	+	+	-
MSBR13	+	-	95	-	+	-	+	+
MSBR14	+	+	100	50	+	+	+	+
MSBR15	+	-	90	-	+	-	+	-
MSBR16	+	-	40	-	+	-	+	-
MSBR17	+	-	50	-	+	-	+	-
MSBR18	+	+	100	35	+	+	+	+
MSBR19	+	-	55	-	+	-	+	-
MSBR20	+	-	60	-	+	-	+	-

*MSBR-maize sugar beet rhizosphere (Observations taken in the same day).

paper and rolled. Seedlings were grown for 15 day at 25°C, and were harvested when the shoots were 35 to 40 cm tall.

Structural diversity of *Pseudomonas spp*. surfactants

The surfactants of the *Pseudomonas* spp. were characterized by high-pressure liquid chromatography (HPLC). Analysis was performed after culturing of all isolates at 20°C for 2 days in 25 ml glass tubes with 3 ml of King's B broth. Samples were obtained by extraction for 1 h with 5 ml of ethyl acetate containing 1% formic acid. The surfactant compounds wereanalyzed by HPLC using a Hypersil BDS C18 column (100 by 4.6 mm; 3 µM particle diameter) held at 40°C, and UV detection (200-400 nM) was performed on a Hewlett-Packard model 1100 HPLC diode array detector. The samples were analyzed in a gradient of 85% eluent B to 100% after 40 min. Eluent flow rate was 1 ml per min. Chromatograms were analyzed using the Hewlett-Packard Chemstation Software package. The identical surfactants were considered when retention times in HPLC chromatograms varied by less than 0.1 min with retention times of one/two major peak.

Statistical analysis

Data were subjected to statistical analysis by following CRD using standard procedure (Steel et al., 1997). The differences among treatment means were compared by applying the Duncan's multiple range tests (DMR) (Duncan, 1955).

RESULTS

CLP producing pseudomonads

The abundance of *fluorescent Pseudomonas* spp. was approximately 5×10^6 colonies per gram of rhizosphere soil sample when tested in two different media. Among the 20 strains, three were selected for their growth performance, CFU/g, fluorescence pigment production (Table 1). When a total of 20 fluorescent pseudomonads were tested for their frequencies of swarming, biofilm assays, 5 isolates were highly variable. Biosurfactant-producing *Pseudomonas* spp. strains were initially screened by drop collapse assay (Table 2).

HPLC analysis

CLP producing *Pf* strains were subsequently verified by HPLC analysis. Peaks (retention time between 27 and 36 min) with the absorbtion spectra at approximately 200 nm (endpoint absorption) were identified as CLP producing *Pf* strains and they were found to be antifungal against major diseases of maize. Three strains were selected based on their color reactions in Hiassorted Rapid Biochemical Identification-Test kit (Table 3) based on

Table 2. Biosurfactant properties of *Pf*-VMD strains.

Strain	Biofilm formation	Swarming	Drop collapse assay	Zoospore motility	Zoospore lysis
Pf-VMD-1	+	+	+	+	+
Pf-VMD-2	+	+	+	+	+
Pf-VMD-3	+	+	+	+	+
Pf-VMD-4	-	-	-	-	-
Pf-VMD-5	-	-	-	-	-
Pf-VMD-6	+	+	+	+	-
Pf-VMD-7	-	-	-	-	-
Pf-VMD-8	-	-	-	-	-
Pf-VMD-9	-	-	-	-	-
Pf-VMD-10	+	+	+	+	+

5 µl droplets of bacterial cell suspensions (OD600 = 1) were tested in a drop-collapse assay on Parafilm; '+', a drop collapse. Zoospore motility was observed microscopically after addition of bacterial cell suspensions (OD600 = 1) to zoospores (10^4 zoospores /ml of Downy mildew sporangia in a 1:1 (v/v) ratio. '+' indicates cessation of zoospore motility. Zoospore lysis was observed microscopically after bacterial cell suspensions (OD600 = 1) were mixed with zoospores (10^4 zoospores/ml) of Downy mildew sporangia in a 1:1 (v/v) ratio. '+' indicates zoospore lysis. Strains were tested for swarming by spotting 5µl bacterial cell suspension (10^9 cells/ ml) on a soft agar (0.6% w/v) plate. A '+' indicates the ability to swarm outwards. Biofilm formation of the bacterial strains was tested in 96-well plates filled with 150µlliquid GS1 medium per well. Biofilms were stained with crystal violet after 48 h of incubation. "+" indicates blue color.

Table 3. Hiassorted rapid biochemical identification-test kit**.**

Test	1	2	3	4	5	6	7	8	9	10
Citrate utilization	+	+	+	+	+	+	+	+	+	+
Lysine utilization	V	V	V	V	V	V	V	V	V	V
Ornithine utilization	V	V	V	V	V	V	V	V	V	V
Urease	+	+	+	+	+	+	+	+	+	+
PAL deamination	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	nd	-	-	-	-	-	-	-	-
H_2S production	V	+	V	V	V	V	V	V	V	V
Glucose	+	+	+	+	+	+	+	+	+	+
Adonitol	+	-	-	-	-	-	-	-	-	-
Lactose	+	-	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	-	-	-	-	-	-	-	-	-

(1-10 = Pf strains used in this experiment); + = Positive (more than 90%); - = Negative (more than 90%); v = 11-89% Positive; nd = No data available.

their sugar utilization and subsequently used for the extraction of VMD for testing their antifungal potential, antiserum production and formulation.

Pf-VMD1 strain belongs to group 1 was colonized well in dual antibiotic selection pressure (Trimethoprim, Streptomycin) and tested against maize pathogens under field conditions.

Antifungal assay

Among the biosurfactant producing *Pf* strains from Maize Sugar Beet Rhizosphere, three strains with maximum CFU/g were selected for testing for their antifungal potential against maize pathogens. The strain *Pf*-VMD1 exhibited highest antifungal activity against maize diseases under *in vitro* conditions (Figure 1). Production of metabolites, change in color of the media was observed in the Pf-VMD1 strain, when they were grown in dual culture against the pathogens (Data not shown). Downy mildew zoospores treated with Pf-VMD1 strain were lysed within 90 s at concentrations of 10^4 zoospores/ml (Figure 3).

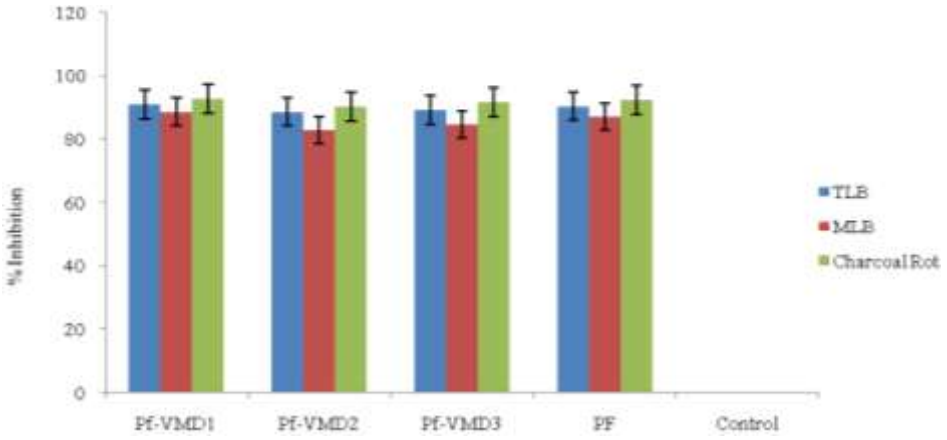

Figure 1. *In vitro* evaluation of VMD producing *Pf* strains against Maize blights (Turcicum Leaf Blight, Maydis Leaf Blight) and charcoal rot.

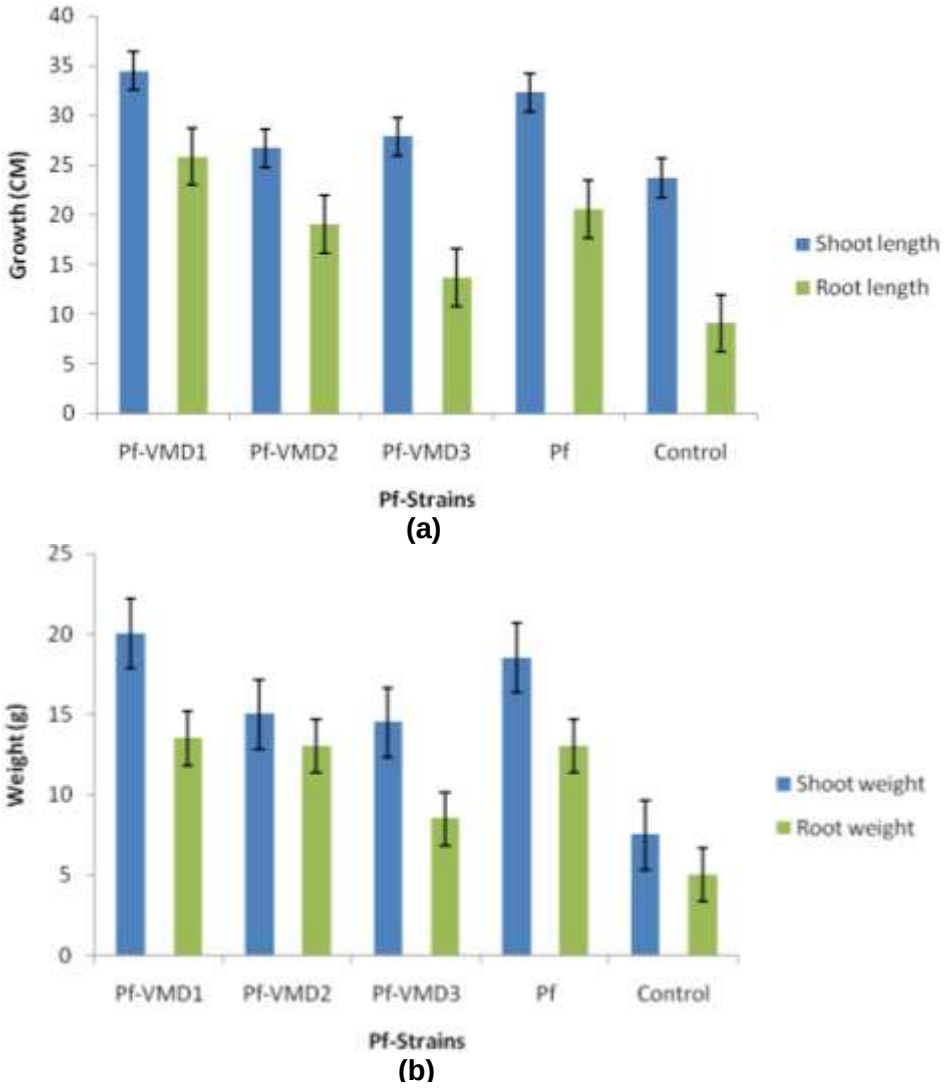

Figure 2. Effect of VMD producing *Pf* strains on growth parameters of maize.

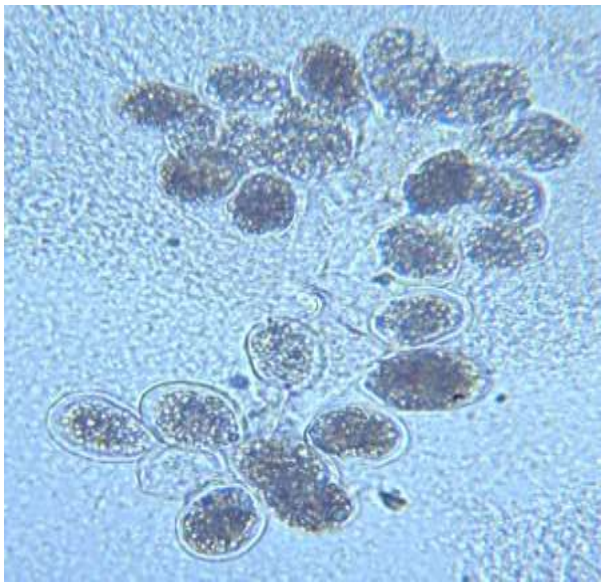

Figure 3. Non motile and lysed zoospores inside the sporangia treated with Pf-VMD1 strain.

Table 4. HPLC analysis of compounds (CLPs) from *Pf*-VMD strains.

VMD group	Retention time	Molecular weight (App.)	Pf-Biotype
V1	32.097,32.007	1125	A
V2	31.814,31.741	1124	B
V3	31.892	1124	B
V4	31.821	1124	B

Growth promotion

The *Pf* VMD1 strain improved the seedling growth, when applied as seed treatment. Increased root lengths with numerous lateral roots were observed (Figure 2). These isolates inhabitants of loamy sand soil were able to produce CLPs. The data further indicating that the soil type may be important for the frequency of CLP-producing strains, since they were isolated from sandy loam soil at Maize Research Station, Vagarai based on the findings of Nielsen and Sorensen (2003).

DISCUSSION

Biosurfactants are amphipathic molecules with a hydrophilic and a hydrophobic moiety, that localize preferentially at the interface between fluid phases with different degrees of polarity such as oil/water or air/water interfaces. Swarming and biofilm assays in the present study proved the presence of such compounds in Pf-VMD strains isolated from sugar beet/maize rhizosphere soil. Lipopeptides form an important group of biosurfactants which are produced by a large variety of bacteria from different genera such as *Bacillus, Lactobacillus, Streptococcus, Serratia, Burkholderia,* and *Pseudomonas* (Velraeds et al., 2000; Mireles et al., 2001; Huberet et al., 2002). Several chemical and biological aspects of CLP production in fluorescent pseudomonads has been discussed by Nybroe and Sorensen (2004). In a recent review, Raaijmakers et al. (2010) have highlighted the structural diversity and activity of CLPs produced by plant-associated *Pseudomonas* spp. Many of the CLPs have 9 or 11 amino acids in the peptide ring with a C_{10} fatty acid at one of the amino acids (Nielsen et al., 2002). HPLC analysis of purified compounds confirmed the presence of Viscosinamide (Based on the retention time between 27 and 36 min) in this present study.

The “V” group are assigned under Viscosinamide-like compound producers, will produce CLPs with MW value of approximately 1120 to 1125 with the retention time

between 31 and 33 min. We have observed 5 out of 20 surfactant- producing isolates form one single group V1, since one major surfactant peak was present in all the isolates. The V1 group surfactant viscosinamid was produced by 25% of the strains (Table 4). We found an interesting result of CLP producing fluorescent Pseudomonads inhabiting maize/sugar beet intercropping in sandy loam soils. Latour et al. (1996) reported that the diversity of CLP producing microbes was mainly influenced by soil type and less by crop type. Similar findings were observed by Bachmann and Kinzel (2001). Apart from these factors, Hoper et al. (1995) suggested that basic soil characteristics such as pH and texture may influence the density of fluorescent pseudomonads. Based on the length and composition of the fatty acid chain as well as the peptide chain, CLPs of *Pseudomonas* species were classified into four major groups, that is, the Viscosin, amphisin, tolaasin, and syringomycin groups (Raaijmakers et al., 2010). The Viscosin class harbours CLPs with 9 amino acids and *Pseudomonas* sp. producing this class of CLPs originate from diverse environmental niches including soil, rhizosphere, phyllosphere, as well as marine environments (Raaijmakers et al., 2010). *Pseudomonas*-derived CLPs are currently divided in eight different structural groups that differ in length and composition of the oligopeptide and fatty acid tail (Olorunleke et al., 2015). The CLPs from the syringomycin class show structural similarity with Viscosin group but contain unused amino acids including Dhb, or 2,4-diamino butyric acid and the lactose ring is formed between the N-terminal and the C-terminal amino acids whereas the ring is formed between the C-terminal amino acid and the 3rd amino acid in the peptide moiety for Viscosin.

In this study, CLP production in *Pseudomonas* spp. isolates from the maize rhizosphere, the exclusive assignment of Viscosinamide production (group V1) *P. fluorescens* biovar I was reported. Membrane interaction and pore formation are often assumed to lie behind the antimicrobial activities of these molecules (Lo Cantore et al., 2006). Pore formation has also been suggested as the mechanism responsible for the adverse effect of Viscosinamide on zoospores of maize downy mildew pathogen (NeilsGeudens et al., 2014).

In a search for the fungal inhibition action of Viscosinamide, Thrane et al. (1991) found that the compound inhibits growth by formation of ion-channels in the fungal membrane. This phenomenon has sub-sequently been confirmed by challenging an *Aspergillus awamori* transformant expressing the Ca^{2+} -sensitive protein aequorin with viscosinamide. The fungus responded to the viscosinamide by a large and immediate increase in cytoplasmic Ca^{2+} -level. Warburton and Deacon (1998) have shown that the permeability of zoospores of *Phytopthora parasitica* increased due to intake of Ca^{2+} just before encystment, resulting in higher intracellular Ca^{2+} levels could thus explain why viscosinamide triggered instant encystment of the fungal zoospores on non-plant surfaces in this study. Compounds with surfactant properties have been successfully deployed in hydroponic systems to control zoospore-producing fungal pathogens (Stanghellini et al., 1997). Apart from the antifungal action of Viscosinamide, it is also involved in the primary metabolism, cell proliferation and strongly binds to the producing cells of the strain DR54 (Nielsen et al., 2000). The findings of the present research also emphasize the above said informations on Viscosinamide-like compounds.

Since, the CLP producers are having synergistic effect of surface motility and the synthesis of antifungal compounds; they could efficiently check and terminate growth of pathogen and could prevent the plants from infection by the pathogen (Koch et al., 2002; Alsohim et al., 2014).

Conclusion

Antimicrobial biosurfactant producing fluorescent Pseudomonads biovar (Pf-VMD1) was isolated from maize/sugar beet rhizosphere in sandy loam soil and tested for the presence of viscosinamide by HPLC analysis. The strain performed its antifungal activity against major fungal disease of maize with zoosporicidal activity against downy mildew pathogen of maize. The strain is grouped under V1 (Viscosinamide-like compounds producers).

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Extended spectrum-β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* causing urinary tract infection

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Extended spectrum β-lactamase (ESBL) producing Escherichia coli and *Klebsiella pneumoniae* causing urinary tract infection (UTI) constitutes a significant therapeutic challenge. The aim of this study was to investigate the frequency of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from urine samples. Clinical isolates of multidrug resistant Gram negative rods (n=102) were taken from urine specimens from tertiary care hospital Lahore. Micro-organisms were identified on the basis of their morphology, cultural characteristics, and confirmed using API 20E. Identified *E. coli* and *K. pneumoniae* were subjected to susceptibility testing using Kirby-Bauer disc diffusion method as per CLSI guidelines. Furthermore, phenotypic confirmation of ESBL production was done according to CLSI guidelines. Out of 102 multidrug resistant Gram negative rods 97 (95%) were ESBL producers isolated from different urine specimens from different wards of hospitals (emergency, ICU, urology ward); among these 64 (66%) were *E. coli* and 33 (34%) were *K. pneumoniae*. Antimicrobial susceptibility pattern of ESBL producing pathogens showed that 75% of these pathogens were resistant to ciprofloxacin whereas most effective drug was meropenem and pipracillin+tazobactam. Meropenem could be used as an alternative treatment option for UTI related to ESBL producing *E. coli* and *K. pneumoniae*. High prevalence of these ESBL producing pathogens are alarming and need special consideration.

Key words: Extended spectrum β-lactamases, urinary tract infection, *Escherichia coli, Klebsiella pneumoniae.*

INTRODUCTION

Urinary tract infection (UTI) has been reported to affect around more than 150 million people globally every year (Picozzi et al., 2014). High burden of UTI are mainly due to Gram negative rods (GNR) (80 to 85%) among these the leading pathogens are *Escherichia coli* (75 to 95%) and *Klebsiella pneumoniae* (Tanvir et al., 2012; Jamil et al., 2014). It is also the second main cause of nosocomial infections at our setup (Qamar et al., 2014). This is associated with a high risk of morbidity, mortality, extra financial budget and even, fatal consequences (Hasan et

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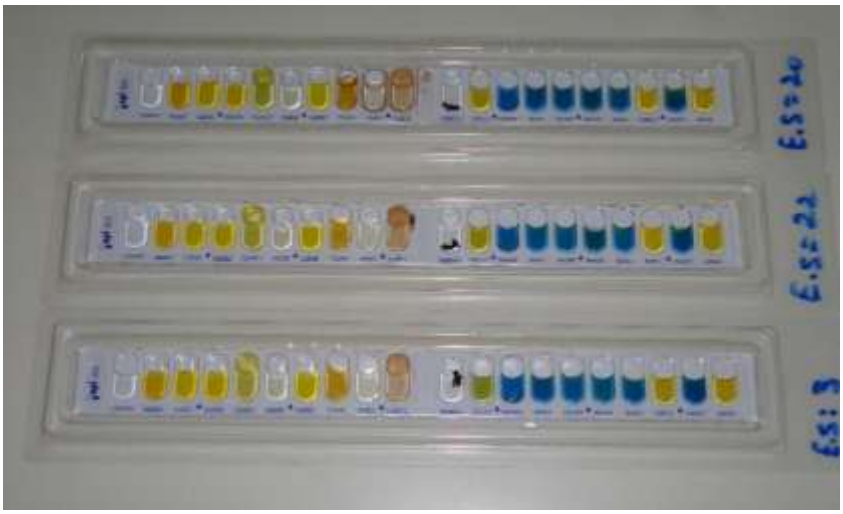

Figure 1. The API 20E used for biochemical confirmation of microorganisms.

al., 2013). World Health Organization and the European Commission has recognized the importance of studying the emergence of different antimicrobial resistant determinants and it is the need to develop appropriate strategies for their control particularly the spread of extended spectrum β-lactamase (ESBL) producing *E. coli* and *K. pneumoniae* (Picozzi et al., 2013). Production of ESBL is a significant resistance-mechanism that impedes the antimicrobial treatment of infections caused by Enterobacteriaceae and is a serious threat to the currently available antibiotic armory (Shaikh et al., 2015). ESBLs are a group of plasmid-mediated, diverse, complex and rapidly evolving enzymes that are posing a major therapeutic challenge today in the treatment of hospitalized and community-based patients (Rawat and Nair, 2010). Reflects the same as previous sentence. They force the physician to use expensive carbapenems to which these are sensitive. The ESBL producers can also develop co-resistance to other classes of antimicrobial agents, such as fluoroquinolones, co-trimoxazole, and aminoglycosides, which are also frequently used for UTI (Potron et al., 2015, Liu et al., 2011). Seriously ill patients with prolonged hospital stays and in whom invasive medical devices are present (urinary catheters, endotracheal tubes) for long duration are at high risk for colonization and infection with ESBL (Peirano and Pitout, 2010). Therefore the aim of this study is to investigate the frequency of ESBL producing *E. coli* and *K. pneumoniae* isolated from urine specimens in a tertiary care hospital Lahore, Pakistan.

MATERIALS AND METHODS

A total of 102 multidrug resistant (MDR) clinical isolates of Gram negative rods were taken from hospitalized UTI patients from different sources (emergency, ICU, urology ward) during December 2013 to December 2014 from tertiary care hospital Lahore.

Identification/purification of isolates

The isolates were culture on blood and MacConkey agar and plates were incubated at 37°C aerobically overnight. The isolates were preliminarily identified on the basis of their morphology and culture characteristics and biochemical profile. Gram-negative isolates particularly *E. coli* and *K. pneumoniae* were further confirmed by API 20E (BioMerieux, France) (Figure 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of isolates was performed by Kirby-Bauer disk diffusion method using Mueller-Hinton agar (Oxoid UK), according to Clinical Laboratory Standards Institute (CLSI) 2012 guidelines (Wikler et al., 2012). The plates were do you mean streaked and inoculated at 35°C for 24 h. Implanted antibiotics were ampicillin (10 µg), cefuroxime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), piperacillin+tazobactam (110 µg), co-amoxeclav (20/10 µg), aztronam (30 µg) and meropenem (10 µg). The interpretation of susceptibility results were done according to CLSI guidelines (Figure 2). Statistical analysis was done using SPSS 22.0.

Screening test was performed using Kirby-Bauer disk diffusion method as per CLSI guidelines (2012). The antibiotic disks of ceftazidime (30 µg), aztreonam (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) were placed on the Mueller Hinton agar (Oxoid) plates per CLSI guidelines. Isolates showing inhibition zone size of ≤ 22 mm with ceftazidime (30 µg), ≤ 27 mm with aztreonam (30 µg), ≤ 27 mm with cefotaxime (30 µg), ≤ 25 mm with ceftriaxone (30 µg) were identified as ESBL positive.

Double disc synergy test ESBL

The double disk synergy test was performed by placing a disk of co-amoxiclav in the center of the plate while ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and cefepime (30 µg) were placed at 20 mm distance center to center from co-amoxiclav disk.

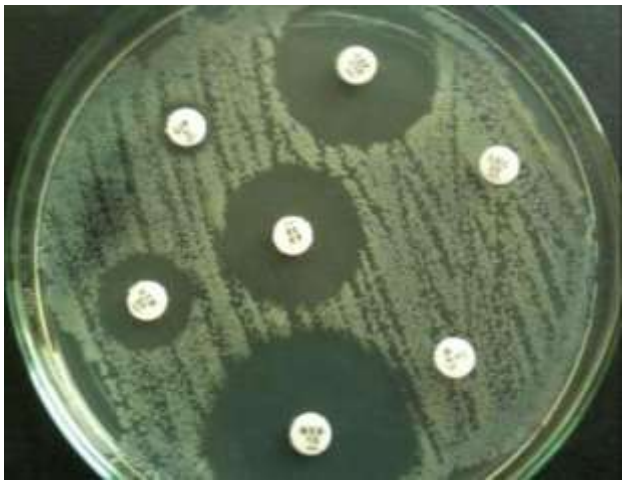

Figure 2. The antimicrobial susceptibility pattern of microorganism screening testing for ESBL.

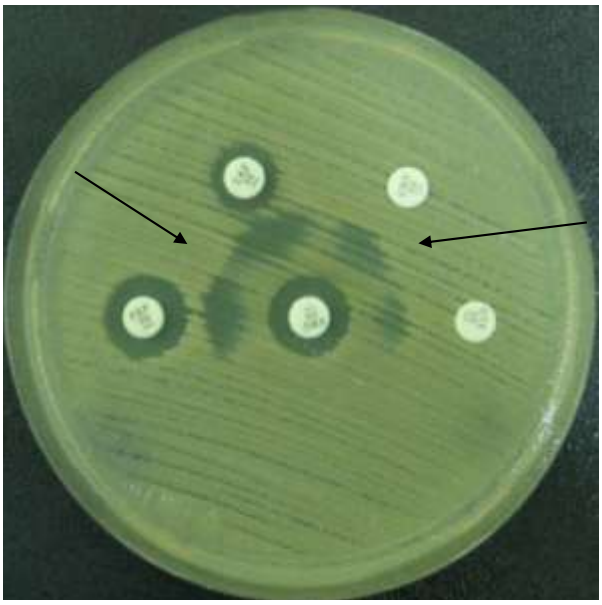

Figure 3. The double disc synergistic method for the confirmation of ESBL producer organisms. The implanted drugs were amoxicillin + clavulanic acid, ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and cefepime (30 µg).

The plates were incubated at 37°C for 18 to 24 h. Isolates which showed zone of inhibition greater than 5 mm on the co-amoxiclav side of the disk compared to the results seen on the side without co-amoxiclav were confirmed as ESBL producers (Figure 3) (Hannan et al., 2013).

RESULTS

Out of 102 clinical isolates; 66 (64.7%) were *E. coli* and 36 (35.3%) were *K. pneumonie*. Among these 97 (95%) were ESBL producers; 64 (66%) were *Escherichia coli* and 33 (34%) were *K. pneumoniae*. Male to female ratio was 1: 2.4 patients (P-value <0.05).

Overall susceptibility pattern of ESBL producing pathogens showed high drug resistance against ESBLs are intrinsically resistant to these drugs, no need to mention here, moderate resistance was observed against ciprofloxacin (75%) whereas most effective drugs was meropenem and pipracillin+tazobactam (Figure 4).

DISCUSSION

Extensive and misuse of β-lactam antibiotics in hospital and community produce a high bacterial resistance which can lead to increased morbidity, mortality and health care costs. During the past decades, ESBL producing *E. coli* and *K. pneumoniae* have emerged as dangerous pathogens both in hospital and community acquired infections due to extensive use of antibiotics particularly third generation cephalosporins. Recent studies revealed that patients with ESBL producing organisms had significantly higher fatality rate than those with non-ESBL isolates (Mehrgan and Rahbar, 2008). The prevalence of ESBL among the clinical isolates varies greatly worldwide and rapidly changing over time. In present study frequency of ESBL producing pathogens was greater in male as compare to female and *E. coli* was the predominant organism. These results are in accordance with the previous studies conducted in Pakistan that reported the high frequency of ESBL producing *E. coli* among females current study shows higher incidence in males, so it is not in accordance with the cited studies (Ejaz et al., 2011, Kausar et al., 2014). This could be due to the limited health care facilities, broad spectrum and misuse of antibiotics and other unhygienic conditions as compared to developed countries and the most sensitive drug was pipracillin+tazobactam and meropenem. This study correlates with the studies done at national level (Ejaz et al., 2011, Hannan et al., 2013). Spread of ESBL in our setting is mainly due to inappropriate infections control practices, contaminated intravenous catheters, feeding tube and various environmental surfaces (door handles, sucker machine, incubators, mattresses, wash basins, floor, sink, emergency trolley, ventilator, ambo bag, laryngeal scopes) and colonized hands of staff. Next major factor is the irrational use of antibiotics, use of broad spectrum antibiotics and substandard practices are very common (Hannan et al., 2013).

Conclusion

It is concluded from the current study that the most effective drugs against ESBL producing pathogens are meropenem and pipracillin+tazobactam and the prevalence such pathogens is gradually increasing in

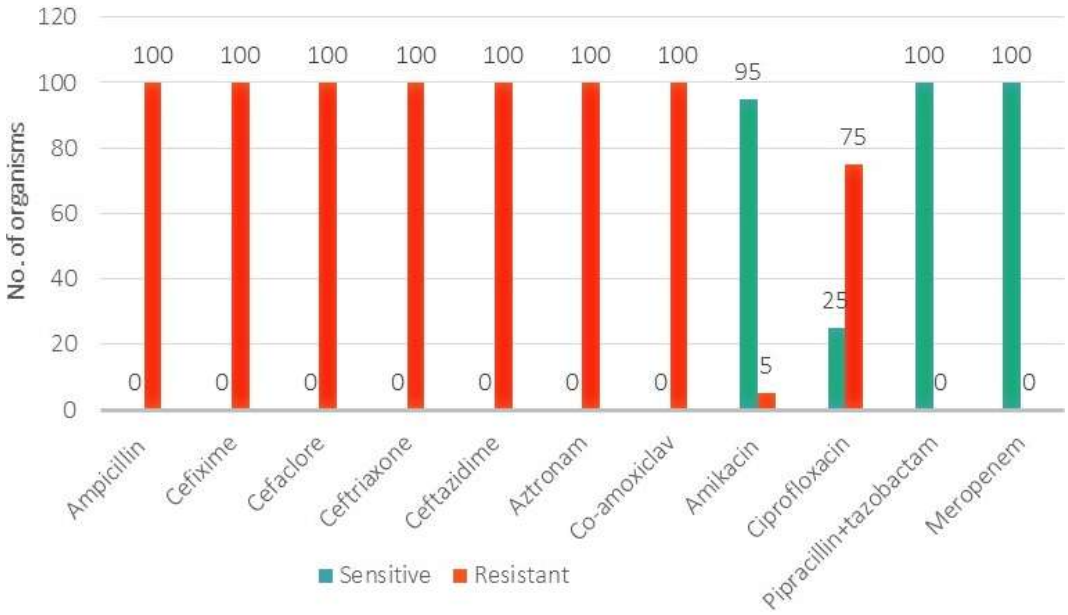

Figure 4. The overall susceptibility pattern of microorganisms; all the microbes showed resistance against commonly drugs except meropenem.

Pakistan and need special consideration.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro antimicrobial activity of three medicinal plants of Ethiopia against some selected bacterial isolates

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Medicinal plants constitute natural source of antimicrobial drugs that will provide essential compounds to fight against disease. In this study, the antibacterial activity of ethanol extracts of *Moringa stenopetala*, *Thymus serrulatus*, and *Terminalia brownii* were investigated against selected pathogenic Gram positive and Gram negative bacteria. *In vitro* antibacterial activities of the ethanol extracts were tested at a concentration of 50, 25, and 12.5 mg/ml by using agar disc diffusion method and zone of inhibitions were determined. Furthermore, minimum inhibitory concentrations were determined for plants that showed antibacterial activity (>15 mm zone of inhibition). The results indicated that only *T. serrulatus* and *T. brownii* exhibited antimicrobial activity against one or more test pathogens. Both extracts of these plants showed strong and dose dependent activity when compared with *M. stenopetala* which demonstrated no activity. Interestingly, *T. serrulatus* showed broad spectrum activity against the tested bacteria. Therefore, ethanol extracts of *T. serrulatus* and *T. brownii* showed promising antimicrobial activity justifying their usage in traditional medicine.

Key words: Antibacterial, Ethiopia, *Moringa stenopetala*, *Terminalia brownii*, *Thymus serrulatus*.

INTRODUCTION

Despite tremendous progress in medicine, infections caused by bacteria, fungi, virus and parasites are still major threat to human and animal health. In the last three decades, few antibiotics were produced but clinical efficacy of these antibiotics is being threatened by the emergence of multi drug-resistant pathogens (Khond et al., 2009). Moreover, antibacterial pharmaceuticals are not accessible to majority of the communities in developing countries (Cheruiyot et al., 2009). Therefore, actions must be taken to reduce these problems, such as controlling the use of antibiotics, understanding the genetic mechanisms of resistance and developing new antibiotics and new therapeutic strategies. Advances in identifying new sources of natural products with antimicrobial activities and expanding antibiotic chemical diversity are providing chemical leads for new drugs

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(Walkty et al., 2014).

Traditionally used medicinal plants produce a variety of compounds for the treatment of various ailments. These medicinal herbs constitute indispensable components of the traditional medicine practiced worldwide due to the low cost, easy access, and ancestral experience; and they are considered as candidates for developing new antimicrobial drugs (Abdalla et al., 2013; Madduluri et al., 2013). Over the past few decades, numerous studies have been conducted on plants to explore possible candidates for antibiotics (Frey and Meyers, 2010). Ethnobotanical studies revealed that wider range of Ethiopian plants are being used in treatment of many diseases in the traditional health care system of the country (Giday et al., 2007; Teklehaymanot et al., 2007). Crude extracts of some Ethiopian plants are known to possess strong antimicrobial activity indicating that these plants can serve as sources of effective drugs against certain microbial agents (Mancini et al., 2015; Taye et al., 2011). *Moringa stenopetala* (bak.) Cuf. (Moringaceae), *Thymus serrulatus* (Lamiaceae) and *Terminalia brownii* (Combretaceae) are among plants which are frequently used in traditional medicine of Ethiopia for the treatment of infectious and non-infectious diseases (Asfaw et al., 2000; Mekonnen and Drager, 2003; Mancini et al., 2015; Wilson and Woldo Gebre, 1979).

M. stenopetala is endemic to East Africa and is mainly present in Southern Ethiopia and Northern Kenya (Padayachee and Baijnath, 2012). This plant is known as Shiferaw in Amharic (Makonnen et al., 1997). Various parts of the plant are used by traditional healers to treat cold, anemia, epilepsy (Demeulenaere, 2001), digestive problems, dysentery, malaria, hypertension, stomach pain, visceral leishmaniasis, asthma, diabetes (Mekonnen and Drager, 2003; Padayachee and Baijnath, 2012) and hyperglycemia (Tesemma et al., 2013). Whereas, *T. serrulatus* is endemic to Ethiopia and it is locally known as Tosign (Asfaw et al., 2000). The leaves and flowering parts of *Thymus* species are widely used as tonic, herbal tea, antiseptic, carminative as well as treating cold (Javadi et al., 2013). *Thymus* spp. are also used as antispasmodic, anti-inflammatory, as expectorants and to treat digestive problems in Iran (Nickavar et al., 2005). On the other hand, *T. brownii* is found in many parts of Africa, such as Democratic Republic of Congo, Ethiopia, Kenya and Tanzania (Fyhrquist et al., 2002). In Ethiopia, it is locally known as Weba. Traditionally, it is used to treat bacterial, fungal and viral infections (Mariod et al., 2014), diarrhea, cut wounds, gonorrhea, cough (Abdalla et al., 2013), jaundice, hepatitis, liver cirrhosis, and yellow fever (Kokwaro, 1976; Wilson and Woldo Gebre, 1979).

Despite of the wide spread uses of these plants as treatment against animal and human infectious diseases, only few report exists on the activity of these plants against micro organisms, such as *Salmonella* species, *Escherichia coli* o15:H7, *Bacillus cereus* and *Staphylococcus aureus.* Therefore, the objective of this study was to evaluate the potential antibacterial activity of these medicinal plants against selected gram positive and gram negative bacteria isolated from samples of animal origin.

MATERIALS AND METHODS

Plant collection and extraction

The leaves of *M. stenopetala*, *T. serrulatus* and *T. brownii* were collected from different parts of Ethiopia. The plants were identified by a botanist and voucher specimens were deposited at the Herbarium of the Addis Ababa University. The leaves of each plant were washed with distilled water and allowed to dry under shade. The dried leaves were ground and extracted with 70% ethanol by maceration in such a way that 150 g of each powdered plant material was soaked in 500 ml of 70% ethanol for 24 h with shaking. The solvent was filtered through Whatman filter paper No. 1 (Whatman, UK), while the residues were used for a second extraction with 300 ml of 70% ethanol. After the second extraction, the filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C and crude extracts thus obtained were stored in refrigerator at 4°C until use.

Preparation of test organisms

S. aureus (from bovine milk), *B. cereus* (from poultry), *Salmonella* spp. (from bovine meat) and *E. coli* o15:H7 (from bovine meat) were isolated and confirmed at the laboratory of Veterinary Microbiology, Hawassa University, Ethiopia according to the standard protocol described in Quinn et al. (1999). Liquid cultures were prepared by placing a loopful of bacteria into 10 ml of nutrient broth grown at 37°C. The turbidity of each liquid culture for use in the assays was then adjusted to 0.5 McFarland standard units using sterile nutrient broth.

Antimicrobial activity assay

The disc-diffusion assay (Bauer et al., 1966) was used to determine the antimicrobial potential of investigated extracts. Methanol was used to dissolve *T. serrulatus* whereas sterile water was used for *M. stenopetala* and *T. brownii*. Extract impregnated discs were prepared in such a way that 100 µl of the extracts prepared at three different concentrations (50, 25 and 12.5 mg/ml) was pipetted onto a 6 mm sterile filter paper disc and allowed to dry overnight at 37°C. For sensitivity test, Muller Hinton agar medium was prepared, poured to each sterile petriplates and allowed to solidify at room temperature. 100 µl of liquid bacterial culture was spread onto the plates. Standard antibiotic discs and extract impregnated sterile discs were then placed on the plates. Each plate contained four paper discs; two discs contained extracts at two different concentrations (either of 50, 25 and 12.5 mg/ml), one disc served as a negative control (100 µl sterile water or methanol impregnated disc), and the other standard antibiotic disc served as a positive control (ceftriaxone (30 µg) for *E. coli* o15:H7 and *Salmonella* spp; tetracycline (30 µg) for *B. cereus* and *S. aureus*). Each extract was tested in triplicate, and the plates were inverted and incubated at 37°C for 24 h. At the end of the incubation period, the antimicrobial activity was evaluated by measuring the diameter of inhibition zones. An inhibition zone of 15 mm or more was considered as high antibacterial activity (Mothana et al., 2009).

Minimum inhibitory concentration (MIC) assay

Based on the screening test, MIC of *T. serrulatus* and *T. brownii* were determined. The agar dilution method recommended by the National Committee for Clinical Laboratory Standards (Prudent et al., 1995) was used with minor modification. A series of two fold dilution of each extract, ranging from 0.125 to 64 mg/ml was prepared in Muellur Hinton agar at 40°C. Plates were dried at room temperature for 30 min prior to spot inoculation with 10 µl aliquots of culture. Inoculated plates were incubated at 37°C for 18 h and the MICs were determined. Experiments were carried out in triplicate. Inhibition of bacterial growth in plates containing test extracts was assessed by comparison with growth in the positive control plates (20 mg/ml of ceftriaxone for *E. coli* o15:H7 and *Salmonella* spp.; 20 mg/ml of tetracycline for *B. cereus* and *S. aureus*). The MICs were determined as the lowest concentration of extract inhibiting visible growth of each organism on the agar plate (Delaquis et al., 2002).

RESULTS

The disk diffusion method for antimicrobial susceptibility testing was initially performed to determine the antibacterial activities of crude ethanol extracts of the leaves of *M. stenoptala*, *T. serrulatus* and *T. brownii* against *B. cereus*, *E. coli* o15:H7, *Salmonella* spp. and *S. aureus. T. serrulates* exhibited concentration dependent antibacterial activity against *E. coli* o15:H7, *B. cereus* and *S. aureus*, whereas *T. brownii* was active only against *Salmonella* spp. At the three concentrations, the minimum zone of inhibition of both plant extracts was 15 mm (Table 1). However, *M. stenopetala* was found to be inactive against all tested bacteria.

The MIC of *T. serrulatus* and *T. brownii* against all tested bacteria are summarized in Table 2. Only those extracts which inhibited the growth of bacterial strains in disc diffusion method were subjected to MIC evaluation. It was found out that *E. coli* o15:H7 was relatively the most susceptible bacteria with the lowest MIC values of crude extracts of *T. serrulatus* (2 mg/ml). On the other hand, MIC of *T. serrulatus* (against *B. cereus* and *S. aureus*) and *T. brownii* (against *Salmonella* spp.) was 4 mg/ml.

DISCUSSION

Plants contain various types of bioactive molecules which are under the targets of extensive research worldwide (Walkty et al., 2014). In the present work, 70% ethanol extract of *M. stenopetala*, *T. serrulatus* and *T. brownii* were subjected to antimicrobial study against *B. cereus, E. coli* 015:H7, *Salmonella* spp. and *S. aureus*. The result of this study shows that the crude extracts of *T. serrulatus* showed concentration dependent inhibition against *B. cereus*, *S. aureus* and *E. coli o15:H7*. At the lowest test concentration (12.5 mg/ml), its potency was comparable to that of standard antibiotics ceftriaxone and tetracycline; and even better than the standard antibiotics at relatively higher concentrations (25 and 50 mg/ml). The antimicrobial activity of *T. serrulatus* found during the current investigation were in agreement with the findings of earlier researchers who determined the antimicrobial activity of aerial parts of related *Thymus* spp. against a wide range of microorganisms (Akrayi and Abdulrahman, 2013; Pirbalouti et al., 2011). In addition, our study results show that *T. serrulatus* did not have activity against *Salmonella* spp. However, some other *Thymus* spp. (*Thymus lanceolatus*) showed inhibitory effect on *Salmonella* spp. (Benbelaïd et al., 2013). The discrepancy may be due to variation in plant parts used, effect of climate, extraction method, and composition of extracted products.

The result of this study also shows that *T. brownii* extracts showed antibacterial activity only against *Salmonella*. This was contrary to an earlier study result where *T. brownii* did not show any activity against salmonella species (Abdalla et al., 2013); however, it has well exhibited activity against *S. aureus*. In addition, *T. brownii* has antibacterial activity against *S. aureus*, *E. coli, Salmonella* and *B. cereus* as reported by Mbwambo et al. (2007). The observed variation might be attributed to the variation in the plant parts and extraction solvent used. Nevertheless, 70% ethanol extracts of leaves of *T. brownii* was used in the present study instead of methanol extracts of barks, wood and whole roots.

In an earlier study, *M. stenopetala* showed antimicrobial activities against *S. auerus*, *E. coli* and *Salmonella* spp. (Tesemma et al., 2013). However, in the present study, the result clearly demonstrated that this plant was devoid of any antimicrobial potential against tested organisms. The reason might be due to the variation in the plant parts and extraction solvents used; where in this study, leaves of ethanol extracts of plant was used instead of acetone extracts of root wood. Other studies on methanol and n-hexane extracts of *M. stenoptala* (Eilert et al., 1981; Walter et al., 2011) and methanol and aqueous extracts of bark and leaf extracts of *M. stenoptala* (Biffa, 2005) revealed that the plant was effective in inhibiting the growth of *E. coli* and *S. aureus*; but only *S. aureus*. Earlier, the compound, namely 4(α-L-Rhamnosyloxy)benzylisothiocyanate isolated from the seeds of *M. stenoptala* showed profound antimicrobial activity against *Mycobacterium phlei* and *B. subtilis* (Eilert et al., 1981).

The discrepancies of these findings with other previous studies are expected as phyto-constituents and are known to vary with ecological factors and seasonal variation (Rafique and Chaudry, 1999). Furthermore, the type of solvent and different phytoconstituents might have played a role (Thaker and Anjaria, 1986). Such factors are known to cause negative or positive effects on the treatment of test microorganisms. It is worth to mention that the antimicrobial components of a plant might have changed in concentration with the age of the plants (Mangla and Kamal, 1989). Environmental factors

Table 1. Mean zone of inhibitions (mm) of three plant extracts against test bacteria.

Bacteria	Mean zone of inhibitions										
	T. serrulatus (mg/ml)			*T. brownii* (mg/ml)			*M. stenopetala* (mg/ml)			Control	
	50	25	12.5	50	25	12.5	50	25	12.5	±ve	-ve
Salmonella species	-	-	-		21.5±0.5	16.5±0.5	15.5±0.5	-	-	18.0±0	-
E. coli 015:H7	19±0	17.5±0.5	15.5±0.5	-	-	-	-	-	-	18.0±1.0	-
B. cereus	20±0	17.5±0.5	15±1.0	-	-	-	-	-	-	16.0±1.0	-
S. aureus	19.5±0.5	18.5±0.5	15.5±0.5	-	-	-	-	-	-	17.0±1.0	-

Values are mean inhibition zone (mm) ± standard deviation (SD) of three replicates, P<0.05. (± ve): Positive control (Ceftriaxone disc for *Salmonella* species and *E. coli 015:H7*; Tetracycline disc for *B. cereus* and S*. aureus*). (-ve): Negative control: sterile water for *T. brownii* and *M. stenopetala*, methanol for *T. serrulates.* (-): no zone of inhibition.

Table 2. Minimum inhibitory concentration (MIC) values (mg/ml) of ethanol extract of *T. serrulatus* and *T. brownii* against tested bacteria.

Plant species	*B. cereus*	*E. coli* o15:H7	*Salmonella* spp.	*S. aureus*
T. serrulatus	4	2	-	4
T. brownii	-	-	4	-

(-): no zone of inhibition.

like excessive rain fall and drought are also reported to enhance the quality of active compounds or diminish it (Vlachos et al., 1997).

It is concluded that the findings of this study justify the claimed traditional uses of *T. serrulatus* and *T. brownii* to treat various infectious diseases in Ethiopia. The results of the present study warranted to initiate in-depthinvestigation on the antimicrobial potential of these plants especially after fractionation, isolation and characterization of active phytoconstituents.

Conflict of interests

The authors declare that the study was carried out purely with the academic interest and there are no competing interests involved.

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Full Length Research Paper

Enteric fever caused by *Salmonella enterica* serovar paratyphi A: An emerging health concern in Nepal

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Enteric fever is an invasive life-threatening systemic disease caused by the *Salmonella enterica* human-adapted serovars typhi and paratyphi. Increased incidence of infection with *S. enterica* serovar paratyphi A poses a significant health concern in some areas of the world. In this study, the incidence of enteric fever confirmed by isolation of *Salmonella* paratypi A or S. typhi from blood cultures of patients presenting with clinical symptoms was 5.1%. Of the total isolates, 64.13% were *S*. paratyphi A, and 35.87% were *Salmonella* typhi. All isolates were susceptible to amoxicillin, ceftriaxone and cefixime. Conventional antibiotics (ampicillin, cloramphenicol and co-trimoxazole) showed 100% sensitivity rate towards *S*. paratyphi A and 96.9% towards *S*. typhi. Overall nalidixic acid-resistance (NAR) rate was extremely high (92.39%). Nalidixic acid resistant (MIC ≥ 32 µg/ml) *S.* paratyphi A showed increased MICs of the fluoroquinolone than nalidixic acid resistant *S*. typhi ranges from 0.125-8 µg/ml with ciprofloxacin and 0.25-4 µg/ml with levofloxacin and was statistically significant (p≤0.001). Immunization with currently available vaccines against typhoid fever does not provide cross protection against paratyphoid fever. This may contribute to the emergence of paratyphoid fever as the major cause of enteric fever in Nepal and possibly other geographical locations.

Key words: Enteric fever, *Salmonella* paratyphi A, *Salmonella* typhi, fluoroquinolones (FQs), Nepal.

INTRODUCTION

Salmonella enterica serovars typhi (*S*. typhi) and paratyphi (*S*. paratyphi) A, B, C are human restricted bacterial pathogens that cause related systemic disease, collectively called enteric fever, remains a common febrile illness in the developing world including the Indian subcontinent, Southeast Asia, Africa, and, to a lesser extent, South America, with poor standard of hygiene and sanitation (Kathryn et al., 2007; Crump et al., 2010; Kariuki et al., 2004). Current estimates from World Health Organization (WHO) suggest that the global burden of typhoid fever is approximately 21 million cases annually with more than 2,20,000 deaths, and that paratyphoid

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fever causes an additional 5.4 million cases (Crump et al., 2004).

In recent years, the incidence of infection with *S*. paratyphi A is elevated in some regions of the globe (Particularly in South-east Asia). It is accountable for up to 50% of all enteric fever cases, causing more asymptomatic infection than *S*. typhi (Woods et al., 2006; Neupane et al., 2010; Mahapatra et al., 2016). In developed countries, enteric fever is a sporadic disease that occurs mainly in returned travelers from the endemic areas (Lee et al., 2004). In recent years, countries like Japan and United States of America have already experienced increase incidence of *Salmonella* paratyphi A in returned traveler from various endemic regions (Judd et al., 2015; Katanami et al., 2016). In Australia, Among 810 *S.* paratyphi A isolated between1985-2010, 547 isolates originated from India, Indonesia, Bangladesh, Pakistan, Nepal, Cambodia, Thailand, Philippines, Papua New Guinea and Lebanon (Commons et al., 2012). In another study carried out in Sydney, 8 *S.* paratyphi A infections were detected during the period January-June 2011 and the patients were predominantly associated with travels to the Indian subcontinent (Blackstock et al., 2012). Recently, three cases of *S*. paratyphi A infection have been reported in French traveler after trekking in Nepal during monsoon season (Jean et al., 2016). A recent outbreak of *S.* paratyphi A in India, Combodia suggests how this neglected tropical diseases rapidly spread in various geographical region of the globe (Verma et al., 2016; Laura et al., 2015).

In Nepal, higher isolation rate of *S.* paratyphi A during summer has been documented in various studies and has become one of the most common culture isolates from patients with febrile illness (Woods et al., 2006; Acharya et al., 2011; Shirakawa et al., 2006). In a retrospective study, 288 out of 541 blood culture samples from patients with enteric fever collected in Tribhuvan University Teaching Hospital, Kathmandu between January and September, 2004 were serotyped as *S.* Paratyphi A (Pokharel et al., 2006).

FQs like ciprofloxacin and ofloxacin, are relatively inexpensive and well tolerated, considered as the most selected groups of antimicrobial for the treatment of uncomplicated enteric fever in adults (Chuang et al., 2009). Unfortunately, outbreaks of *S.* paratyphi A strains that were resistant to nalidixic acid (the prototype quinolone, which is used for in vitro screening tests), accomplished reduced susceptibility to the FQs have been reported subsequently in India, Pakistan, China and South East Asia (Parry et al., 2002; Chuang et al., 2009; Hakanen et al., 1999; Threlfall et al., 1999) and infections with elevated MICs to FQs have been related with the treatment failure and increases disease severity (Hakanen et al., 1999; Renuka et al., 2004).

Third generation cephalosporin is associated with higher cure rates in the FQs resistant patients (WHO, 2003). However, resistance in third generation cephalosporin from the different parts of the world in *S*. paratyphi A, is an ever increasing problem, and is a cause of serious concern for the treatment of enteric fever (Pokharel et al., 2006; Vincet et al., 2008; Nashwan et al., 2008; Morita et al., 2010; Roya et al., 2015).

MATERIALS AND METHODS

Study area

This study was carried out at microbiology laboratory of Nepal Medical College Teaching Hospital, Kathmandu on clinically defined suspected enteric fever cases requesting for blood culture and antibiotic susceptibility testing from March 2012 to September 2012. A total of 1803 blood samples from the febrile ill patients were included in this study.

Microbiology

Blood samples were collected aseptically by venipuncture and inoculated immediately into brain heart infusion broth and incubated at 37°C for 24 h. After incubation, subculture was done on MacConkey agar and Blood agar. Identification of positive culture plates was carried out with the standard microbiological procedure including colony morphology, staining reaction, biochemical characteristics and serotyping using specific antisera (Denka Co. Ltd, Tokyo, Japan). Samples were considered negative for *Salmonella* if no growth was observed until 10 days of incubation (Cheesbrough, 20005).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method with Mueller-Hinton agar using the guidelines and interpretive criteria of the CLSI (CLSI, 2011). Antibiotic discs: ampicillin (10 µg), chloramphenicol (30 µg), cotrimoxazole (1.25/23.75 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), gatifloxacin (5 µg), cefixime (30 µg), cefepime (3 µg), ceftriaxone (30 µg) were tested for all confirmed isolates. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Determination of minimum inhibitory concentration (MIC)

MICs of nalidixic acid, ciprofloxacin, levofloxacin were determined by agar dilution method following CLSI 2011 guideline. *Escherichia coli* ATCC 25922 was used as the quality control strain.

Ethical clearance and consent

Written consent form was obtained from the Institutional Research/ Review Committee (IRC) Nepal Medical College Teaching Hospital at the time of enrollment, prior to commencing the laboratory work and final report was submitted to research and review committee.

Statistical analysis

Statistical analysis was performed using WHONET 5.6 and SPSS 19 software, Student t-test and Chi-square test were used to determine the significant confidence interval (P-value).

Table 1. Month wise distribution of *S*. typhi and *S*. paratyphi A.

Salmonella	Month							Total
	March	April	May	June	July	August	September	
Febrile cases	155	231	302	314	361	242	198	1803
S. typhi	-	3	10	7	1	6	6	33
S. paratyphi A	-	13	24	10	3	6	3	59
Total cases	-	16	34	17	4	12	9	92

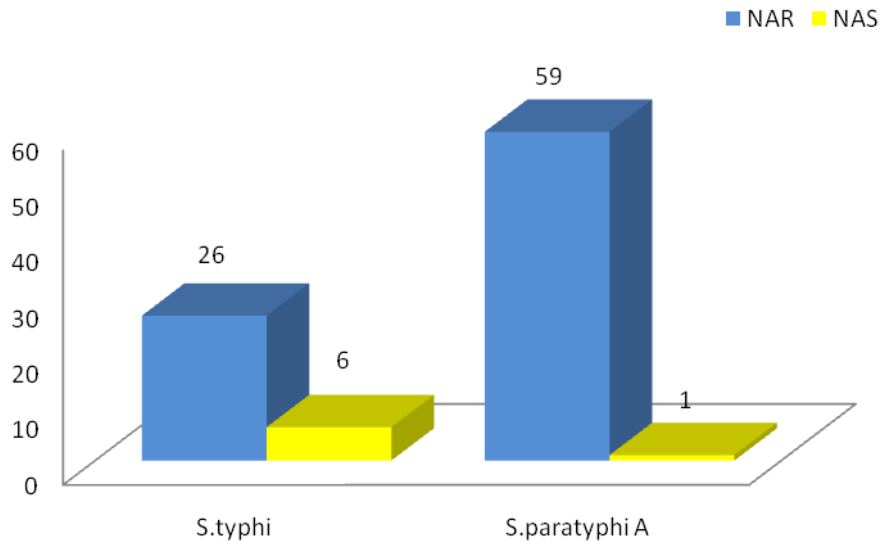

Figure 1. Nalidixic acid resistance pattern in *S*. typhi and *S*. paratyphi A.

RESULTS

A total of 1803 blood culture samples from patients with febrile illness visiting Nepal Medical College Teaching Hospital, Kathmandu, were included in this study. A total of 92 (5.1%) of the blood culture samples were positive for *Salmonella enterica* growth. Serotyping showed that out of 92 isolates, 59 (64.13%) were *S*. paratyphi A and 33 (35.87%) were *S*. typhi. The distribution of these serotypes in age groups varied from 45 days child to 65 years old man with the mean age group of growth 20.59 years. Out of total positive isolates: 62 (67.4%) cases from male, and 30 (32.6%) cases from female (P<0.05). Most of the enteric fever cases were found in the month of May (11.52%) of the total suspected cases. The number of *S*. paratyphi A increases significantly in each month but in August, equal number of *Salmonella* was isolated and in September number of *S*. typhi were greater than the number of *S*. paratyphi A (Table 1).

Antimicrobial susceptibility to quinolone showed that 7 (7.6%) isolates were susceptible and 85 (92.39%) isolates were resistant to nalidixic acid (no zone of inhibition in 30 µg disc). Resistance to nalidixic acid in *S*. typhi and *S*. paratyphi A was 81.81 and 98.30%, respectively (P=0.008) (Figure 1). Overall, nalidixic acid resistance (NAR) was extremely high (92.39%). Among the FQs, newer FQs like gatifloxacin and levofloxacin equally showed highest sensitivity rate (97.82%) followed by ofloxacin (92.39%), ciprofloxacin (88.04%). However, susceptibility to conventional antibiotics (ampicillin, chloramphenicol and co-trimoxazole) was 100% in *S*. paratyphi A and 96.9% in *S*. typhi. Only one isolates (*S*. typhi) (1.08%) was MDR strain. All isolates showed similar sensitivity rate (100%) towards ceftriaxone and cefixime. Similarly, amoxiclillin yielded 100% sensitivity rate towards both *S*. typhi and *S*. paratyphi A, whereas azithromycin displayed 45.5 and 27.12% sensitivity rate towards *S*. typhi and *S*. paratyphi A, respectively. Intermediate strain should be further evaluated by the MIC determination (Table 2).

MIC of quinolone

Of the total isolates, 85 (92.39%) have nalidixic acid MIC of ≥ 32 µg/ml and were classified as resistant, while 7

Table 2. Minimum inhibitory concentration (MICs) of quinolone antimicrobial agents against *Salmonella enterica* isolates by agar dilution method (N= 92).

Serotype	Antibiotics								
	Nalidixic acid			Levofloxacin			Ciprofloxacin		
	S (≤16 µg/ml)	I	R (≥32 µg/ml)	S (≤2 µg/ml)	I (4 µg/ml)	R (≥8 µg/ ml)	S (≤1 µg/ml)	I (2 µg/ml)	R (≥4 µg/ml)
S. enterica typhi(*N*=33)	6	-	27	31	2	-	31	-	2
S. enterca paratyphi A (N=59)	1	-	58	59	-	-	50	9	-

S, Susceptible; I, Intermediate; R, Resistant.

Table 3. Antibiotic susceptibility pattern of *Salmonella enterica* serotypes typhi and paratyphi A by Kirby-Bauer disc diffusion method.

Antiboitic	Serotype typhi (N=33)			Serotype paratyphi A (N=59)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin	32 (96.97)	-	1(3.03)	59 (100)	-	-
Chloramphenicol	32 (96.97)	-	1(3.03)	59 (100)	-	-
Cotrimoxazole	32 (96.97)	-	1(3.03)	59 (100)	-	-
Nalidixic acid	6 (18.18)	-	27(81.82)	1 (1.70)	-	58 (98.3)
Ciprofloxacin	30 (90.91)	1(3.03)	2(6.06)	47 (79.66)	12 (20.34)	-
Ofloxacin	31 (93.94)	-	2(6.06)	54 (91.5)	5 (8.48)	-
Levofloxacin	31 (96.87)	2(9.1)	-	59 (100)	-	-
Gatifloxacin	33 (100)	-	-	59 (100)	-	-
Ceftriaxon	33 (100)	-	-	59 (100)	-	-
Cefixime	33 (100)	-	-	59 (100)	-	-
Azithromycin	15 (45.55)	12(36.36)	6(18.19)	16 (27.12)	29 (49.15)	14 (23.73)
Amoxicillin	33 (100)			59 (100)		

S, Susceptible; I, intermediate; R, resistant.

(7.6%) have MIC of ≤ 8 µg/ml and were classified as susceptible. For ciprofloxacin, 81 (88.04 %) isolates have MIC of ≤ 1 µg/ml and were classified as susceptible, while 9 (9.78%) has MIC of 2 µg/ml, and was classified as intermediate, while 2 (2.17%) has MIC of 4 µg/ml and was classified as resistant. Similarly, for levofloxacin, 90 (97.82%) isolates have MIC of ≤ 2 µg/ml, and classified as susceptible, while 2 (2.44%) have MIC of 4 µg/ml, and classified as intermediate according to CLSI recommendation criteria (Table 3). Based on NA susceptibility, the MIC of ciprofloxacin and levofloxacin for susceptible isolates showed bimodal distribution. MIC of ciprofloxacin ranges from <0.004 to 0.004 µg/ml in NAS isolates, whereas, 0.125 to 1 µg/ml in NAR isolate (Figure 2). Similarly, MIC of levofloxacin ranges from 0.004 to 0.06 µg/ml in NAS isolates, and 0.25 to 2

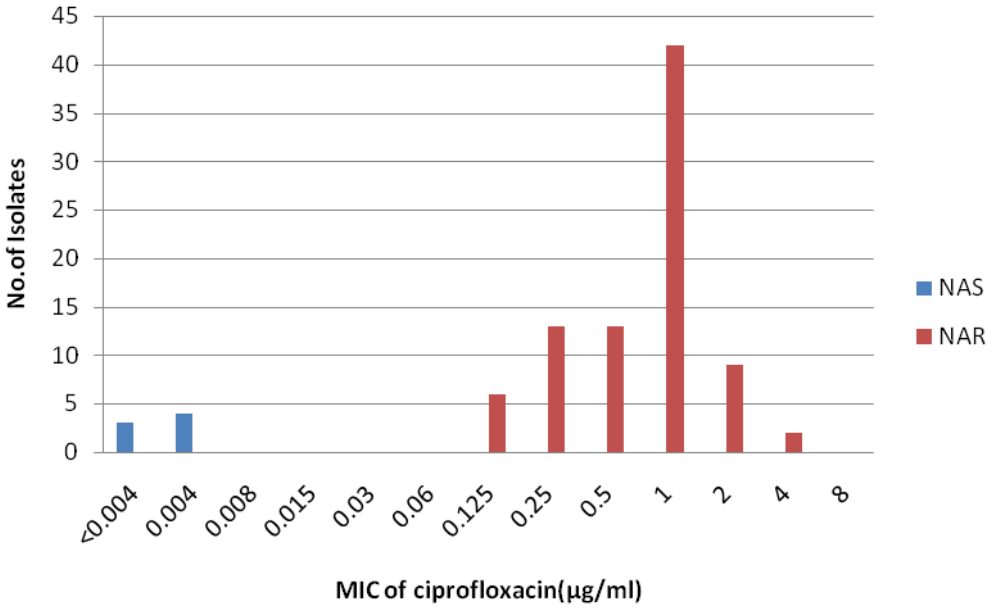

Figure 2. Distribution of MIC of ciprofloxacin between NAR and NAS isolates.

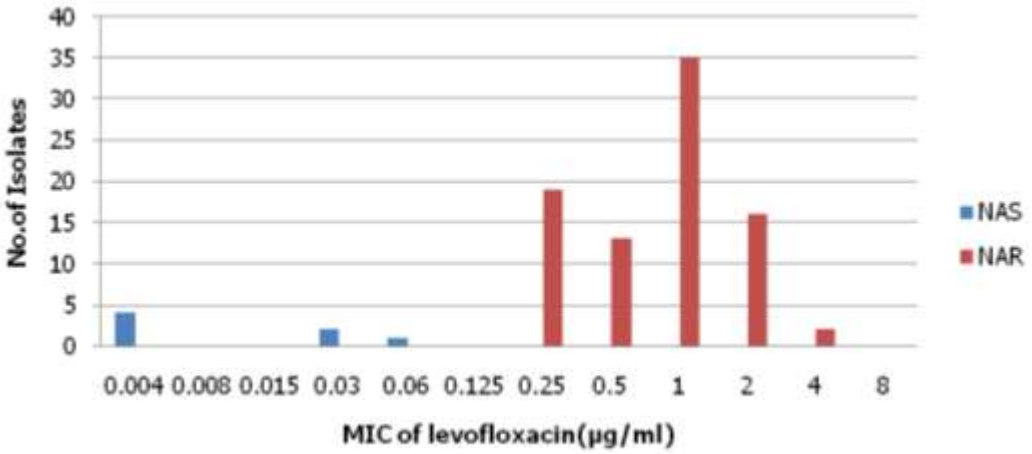

Figure 3. Distribution of MIC of levofloxacin between NAR and NAS isolates.

µg/ml in NAS isolates (Figure 3).

The scatter plots correlate the MICs of levofloxacin and ciprofloxacin with nalidixic acid; demonstrate the simultaneous presence of nalidixic acid resistance and reduced levofloxacin and ciprofloxacin susceptibility. When ciprofloxacin MIC of ≥ 0.125 µg/ml was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥32 µg/ml) led to detection of all 85 isolates with reduced ciprofloxacin susceptibility and none of the susceptible isolates. Thus, the sensitivity and specificity of the validity of nalidixic acid screening approach was 100 and 92%, respectively. Similarly, when an levofloxacin MIC of ≥ 0.25 µg/ml was adopted as a breakpoint, screening for nalidixic acid resistance (MIC ≥ 32 µg/ml) led to the detection of all 85 isolates with reduced levofloxacin susceptibility (MIC ≥ 0.25 µg/ml) and none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100 and 93%, respectively.

Of the 81 ciprofloxacin susceptible isolates, 74 revealed reduced susceptibility to cirprofloxacin (MIC ≥0.125 µg/ml). Similary, levofloxacin also showed reduced susceptibility (MIC ≥0.25 µg/ml) to 83 levofloxacin susceptible isolates. The mean quinolone MICs in Nalidixic resistant *S.* typhi and *S.* paratyphi A was statistically significant (P<0.001). The NAR *S.* Paratyphi A required increased MICs of the FQs in comparison with

the NAR *S.* typhi. The difference in mean FQs MIC in NAR *S.* typhi and NAR *S.* paratyphi A was statistically significant (P=0.001).

Based on scatter plot analysis, to accommodate a susceptible MIC of ≤1 µg/ml, the zone diameter of 5 µg ciprofloxacin disc for susceptible organism increased to about 24 from 21 mm with corresponding increase in zone diameter for resistant from ≤15 to about 23 mm for resistant MIC of ≥4 µg/ml. Similarly, to accommodate a susceptible MIC of ≤2 µg/ml, the zone diameter of 5 µg levofloxacin disc for susceptible organism increased to about 19 from 17 mm with corresponding increase in zone diameter for resistant from ≤13 to about 17 mm for resistant MIC of ≥8 µg/ml.

DISCUSSION

The shifting of *Salmonella* infection with *S*. Paratyphi A from *S*. typhi, antimicrobial resistance pattern, new approaches to treatment and control strategies, are the rising issues. In this study, the overall growth positivity rate of enteric fever was 5.1%, which is low as compared to the previous studies conducted in Nepal (Malla et al., 2005; Maskey et al., 2008; Shirakawa et al., 2006; Fangtham et al., 2008). Lack of growth in blood culture is common in Nepal mainly because of the use of antibiotics prior to blood collection for culture and moreover misuse of antibiotics even for mild cases of fever is common. More importantly, self-medication is widespread with antibiotics freely available without a prescription (Gupta et al., 2009; Ochiai et al., 2005; Lunn et al., 2010).

Out of 92 *Salmonella* positive cases, 59 (64.13%) were *S*. paratyphi A and 33 (35.87%) were *S*. typhi; indicating higher prevalence of paratyphoid cases than typhoid cases. This finding is higher than the earlier report in Nepal (Shirakawa et al., 2006; Maskey et al., 2008; Pokharel et al., 2009; Acharya et al., 2011). Based on the report from various parts of the world, an estimated one case of paratyphoid fever occurs for every four cases of typhoid fever (Crump et al., 2007). It was reported that in Kathmandu, Nepal, enteric fever caused by *S*. paratyphi A is more prevalent than that caused by *S*. typhi (Shirakawa et al., 2006).

A five year (1994-1998) retrospective analysis at New Delhi in India showed rise in proportion of S. paratyphi A from 6.5 to 44.9%, whereas in Calcutta, isolation rate of S. paratyphi A was 11.1% in 2001 and rocketed to 59% in 2003 (Gupta et al., 2009). Likewise, during 2008–2012 in United States, 2341 enteric fever cases were reported; 80% typhoid and 20% paratyphoid. The proportion caused by paratyphoid A increased from 16 (2008) to 22% (2012) (Date et al., 2016). Nigeria reports the most comprehensive data on *S*. paratyphi from sub Saharan Africa. Nigeria has reported that up to 34 % of enteric fever cases are caused by *S*. Paratyphi A (Akinyemi et al., 2007). Since the past decade, the incidence of *S*. paratyphi A has increased worldwide, moreover in south-central Asia and Southeast Asia countries, it appears to be responsible for up to 50% of blood stream infection (Ochiai et al., 2005; Fangtham et al., 2008). These incidences suggest how rapidly paratyphoid fever increase in various parts of the globe.

Change in host susceptibility, change in virulence of the organism and wide spread use of vaccines and quinolones against S. typhi in the past decade might be major causes of higher proportion of *S*. Paratyphi A in recent years (Gupta et al., 2009; WHO, 2003). In addition to this, in recent years, increased popularity of street food consumption that is also a known risk factor for acquisition of *S.* paratyphi A has been shown (Vollaard et al., 2004). There is a proposed reason that SPA infection is related to higher inocula and ST involves small inocula as food borne transmission is associated with large inocula (Crump et al., 2010).

In this study, rate of NAR, which is a phenotypic marker for reduced susceptibility to fluoroquinolones (Hakanen et al., 1999), was very high (92.39%). *S.* paratyphi A strains showed even higher rate (98.30%) of NAR than *S.* typhi (81.81%). Resistance to NA among *S*. Paratyphi A isolates was recently found to be more common than among *S*. typhi isolates obtained from hospitalized patients in Nepal and India (Acharya et al., 2011; Maskey et al., 2008; Shirakawa et al., 2006).

NAR isolates showed reduced susceptibility to FQs (ciprofloxacin and levofloxacin). Nalidixic acid itself is never used for the treatment of typhoid. However, these isolates are susceptible to FQ in disc sensitivity testing according to current guidelines. The clinical response to treatment with FQs of NAR is significantly worse than with NAS strains. FQs treatment failure has also been reported in patients with NAR *Salmonella* infection (Threlfall et al., 1999). The emergence of NAR *S*. paratyphi A strain is worrying given that ciprofloxacin and ofloxacin are the most commonly used antibiotics for the management of enteric fever in Nepal (Lunn et al., 2010). Apart from these reduced susceptibility in *Salmonella* isolates, complete fluroquinolone resistant *Salmonella* isolates pose a new challenge in the management of enteric fever. In a study carried out in Nepal, all the *S*. Typhi and *S*. Paratyphi A isolates were reported as susceptible until 1998 but during 1999 to 2003, ciprofloxacin resistance increased to 5% in the *S*. Typhi and 13% in *S.* Paratyphi A (Maskey et al., 2008). Another study in Nepal revealed five ciprofloxacin resistant and 7 ofloxacin resistant isolate (Bhatta et al., 2005). A recent study in India showed that Ciprofloxacin resistance was observed in 21% (28/133) of isolates by MIC test (Gopal et al., 2016). Elevated level of reduced susceptibility to fluoroquinolone and even some floroquinolone resistant isolates showed that the treatment of the enteric fever cannot rely on the floroquinolones.

Susceptibility to conventional antibiotics was 100% in *S*. paratyphi A and 96.9% in *S*. typhi showing the decline

of MDR strain and re-emergence of susceptible towards these antibiotics. Other studies from Nepal also found susceptibility towards these conventional antibiotics (Acharya et al., 2011; Shirakawa et al., 2006; Gupta et al., 2009). Based on the observation of re-emergence of susceptibility, conventional first line antimicrobials may play vital role in the management of NAR, and non MDR isolates

In this study, third generation cephalosporin showed 100% sensitive rate towards *S*. paratyphi A. However, extended spectrum beta-lactamase (ESBL) producing *S*. Paratyphi A was isolated in Nepal (Pokharel et al., 2006). Likewise, ESBL producing *S*. paratyphi A was isolated in India (Roy et al., 2015). In addition to this, ESBL producing S. Paratyphi A was isolated from a Japanese traveler to Southeast Asia (Mawatari et al., 2013). Increase incidence of *S*. paratyphi A with decrease susceptibility to fluoroqunilone, fluoroquinolone resistant isolate and ESBL producing isolate limit the treatment of enteric fever. Therefore, early preventive measure like vaccination will be vital in the future to prevent spread in travelers in endemic region as well as native of endemic setting. Currently, the two main vaccines recommended for travelers are the Vi polysaccharide vaccine and the oral Ty21a vaccine. These internationally licensed vaccines are safe and effective against *S*. Typhi. However, there is currently no commercially available vaccine against *S*. paratyphi, which is increasingly reported as a cause of enteric fever (Dave et al., 2015). Recently, French Travelers vaccinated by Vi vaccine against typhoid fever returning from Nepal found *S*. paratyphi A infection (Jean et al., 2016). The fact that current typhoid vaccines have no efficacy against *S.* paratyphi A may interfere to expand typhoid vaccination campaigns for regions with a high incidence of confirmed *S.* typhi disease as this is not likely to solve the problem alone or to make a significant contribution if outbreaks are due to *S.* paratyphi A (Wilde et al., 2007).

In conclusion, this study found 5.1% prevalence, reveals enteric fever is still endemic in an urban setting in Nepal, resulting in significant febrile illness. Moreover, increase incidence of S. paratyphi A with decrease susceptibility to fluoroquinolone demonstrates the need to improve water supply and sanitation system to avoid fecal contamination. This increment of paratyphoid fever will be the new threat for the native as well as non-native. Clinician should be on alert with the treatment with fluroquinolones, as patients with enteric fever due to isolates with decreased fluroquinolone susceptibility, are more likely to have prolonged fever clearance time and higher rates of treatment failure. More comprehensive surveillance of antimicrobial resistance among *S*. paratyphi A strains is warranted in Nepal to determine the extent of geographic expansion of resistant strains from Nepal and to inform treatment options for management of patients. A systematic outbreak investigation to determine source and routes of transmission is recommended.

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Conflict of interest

The authors have not declared any conflict of interest

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Full Length Research Paper

Analysis of high levels of multidrug resistant *Escherichia coli* from healthy broiler chickens in Western Algeria

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One hundred and two *Escherichia coli* strains isolated from healthy broiler chickens collected from April 2012 to November 2014 in seven geographic areas of western Algeria were studied. Susceptibility pattern to 11 antimicrobial agents was determined by disk diffusion method as recommended by the Clinical Laboratory Standard Institute (CLSI). Antibiograms revealed high levels of resistance to quinolones and fluoroquinolones including nalidixic acid (100%), flumequin (86, 27%) and enrofloxacin (84, 31%), and to other antibiotics, notably: tetracycline (92, 15%), trimethoprim-sulfamethoxazol (80, 39%) and amoxicillin (68, 62%). However, a moderate percentage of strains were resistant to neomycin (31, 37%), chloramphenicol (27, 45%) and nitrofurantoin (21, 56%). Only 7, 84% of the isolates were resistant to gentamicin and all the isolates were susceptible to colistin. All isolated *E. coli* were resistant to at least three antibiotics. 96, 08% and 91, 12% of the isolates were resistant to at least four and five antimicrobials, respectively. Twenty eight antibiotic resistance patterns of *E. coli* strains were detected, of which 11 were present significantly. The results of this survey indicate very high levels of resistance to quinolones and to other antibiotics in *E. coli* from healthy broiler chickens in Algeria, and suggested that this reservoir of resistance may affect the therapeutic potential of fluoroquinolones in human and veterinary medicine.

Key words: *Escherichia coli*, antibiotic resistance, poultry, Algeria.

INTRODUCTION

Escherichia coli is a common commensal bacterial species that colonizes the gastrointestinal tracts of both humans and animals, but in debilitated or immune suppressed host or after a trauma or surgical procedures, pathogenic and non-pathogenic strains can cause disease in both groups. It can also easily contaminate food products during animal evisceration at slaughter or during food manipulation (Wilhelm, 2011). Antibiotics are

used for therapy and control of bacterial infections in humans and animals as well as for prophylaxis and growth promotion in food producing animals (Sukumaran et al., 2012). Infections with antimicrobial-resistant bacteria are a great challenge in both human and veterinary medicine. Treatment for *E. coli* infection has been increasingly complicated by the emergence of multidrug-resistant bacteria. The emergence of *E. coli* isolates with multiple antibiotic-resistant phenotypes, involving coresistance to four or more unrelated families of antibiotics, has been previously reported and is considered a serious health concern (Maynard et al., 2003). In veterinary medicine, resistance to multiple antimicrobials was found more often in *E. coli* from broiler chickens as compared to *E. coli* from other meat producing animals (Sheikh et al., 2012). Increasing acquired resistance to antibiotics is becoming a worldwide concern and the World Health Organization recommended stopping intensive routine use of antimicrobials in production animals in 2007 (Collignon et al., 2009).

Antimicrobial resistant isolates and resistance genes of *E. coli* can be transferred to humans through the food chain, representing a potential risk for public health (Alexander et al., 2010; Ryu et al., 2012). The main aim of this study was to evaluate the frequency of resistance to quinolones and other groups of antimicrobial agents in *E. coli* isolates from healthy broiler chickens in western Algeria.

MATERIALS AND METHODS

Sample collection

From April 2012 to November 2014, a total of 150 samples from healthy broiler chickens were collected from broiler farms situated in seven geographic areas of Western Algeria including: Mostaganem, Oran, Mascara, Relizane, Chlef, Tiaret and Tissemsilt. Samples were randomly taken from poultry farms and directed to the laboratory for further experimentation.

Bacteriological analysis

Autopsies were performed in the Regional Veterinary Laboratory of Mostaganem, Algeria, and tissue samples from liver, spleen and pericardium were collected. *E. coli* strains were isolated and identified according to the method previously described by Livrelli et al. (2007). Briefly, for the primary isolation of *E. coli,* visceral organs were cut into small pieces using sterile scissors. Enrichment was done by seeding cubes in tubes of BHI broth (Pasteur Institute of Algeria) and incubated aerobically overnight at 37°C. A drop of broth was inoculated with BHI broth method of exhaustion on MacConkey agar medium (Pasteur Institute of Algeria) and then incubated at the same time and temperature as described previously. For each sample, one colony with typical *E. coli* trait was picked and re-isolated on MacConkey agar and the phenotypic identification result was confirmed by classical biochemical testing or using the API 20E system (BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all *E. coli* isolated strains was tested according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). The isolates were tested against a panel of 11 antimicrobials: nalidixic acid (NA, 30 µg), flumequin (UB, 30 µg), enrofloxacin (ENR, 5 µg), amoxicillin (AMX, 25 µg), tetracycline (TE, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1, 25/23, 75 µg), Neomycin (N, 30 µg), gentamicin (CN, 15 µg), chloramphenicol (C, 30 µg), Nitrofurantoin (FT, 300 µg) and colistin (CT, 50 µg). Antibiotic disks were purchased from Bio-Rad (Marnes la Coquette, France). Results were obtained after incubating samples for 16 to 18 h at 37°C and were interpreted according to CLSI previously cited guidelines. *E. coli* ATCC 25922 was used as a quality control strain.

RESULTS AND DISCUSSION

The emergence and spread of multidrug-resistance, including resistance to first-line treatment drugs, in bacteria from food producing animals is a major therapeutic problem. Two hundred and two *E. coli* strains isolated from 150 samples from healthy broiler chickens sampled a few days before slaughter were included in this study. The frequency of resistance of each antibiotic tested is shown in Figure 1. In the current study, *E. coli* isolates showed a high resistance rate to quinolones and fluoroquinolones, notably nalidixic acid (100%), flumequin (86, 27%) and enrofloxacin (84, 31%). Resistance to these antibiotics were far higher as compared to other studies in the same region (Hammoudi and Aggad, 2008; Aggad et al., 2010). But, Moniri and Dastehgoli (2005) in their study of 181 *E. coli* isolated from healthy broilers in Iran reported also that 100% of the isolates were resistant to nalidixic acid; however, less resistance to fluoroquinolones was observed (Moniri and Dastehgoli, 2005). The high recovery rate of fluoroquinolone-resistant *E. coli* from broilers in Algeria was troubling, but not surprising, given the routine application of the quinolone (nalidixic acid and flumequine) at subtherapeutic doses for prophylactic and therapeutic purposes in broilers, and the fluoroquinolones (enrofloxacin) for the prevention of early chick mortality and to reduce possible colonization or horizontal spread of pathogens after day-old broiler

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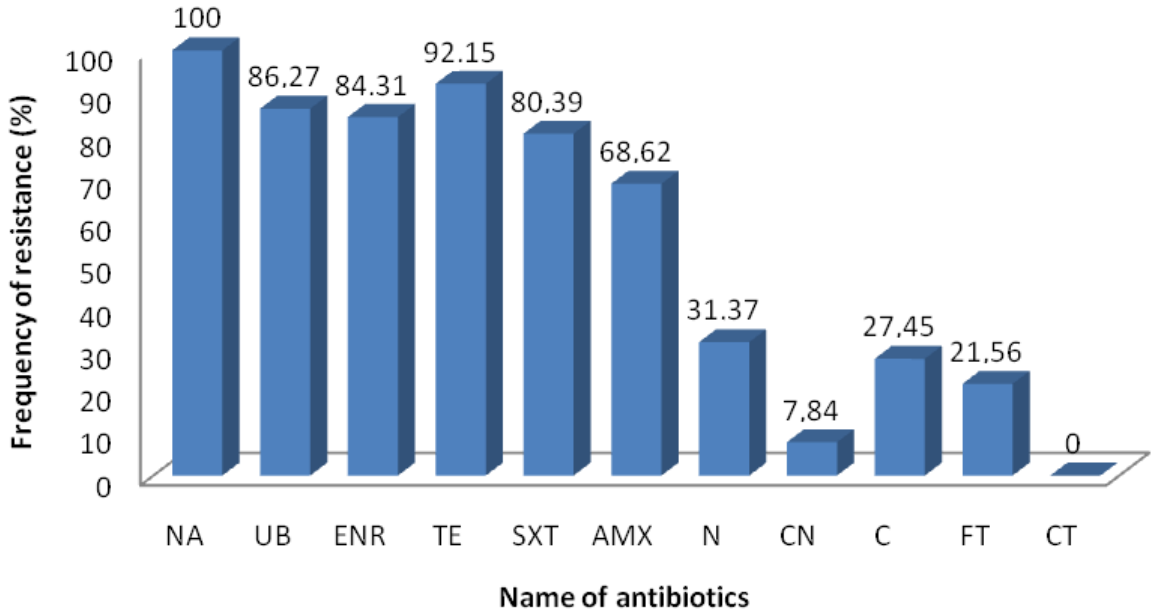

Figure 1. Percentage of antibiotic resistance among *E. coli* strains isolated from healthy chickens. NA: Nalidixic acid; UB: flumequin; ENR: enrofloxacin; TE: tetracycline; SXT: trimethoprrim-sulfamethoxazol; AMX: amoxicillin; N: neomycin; CN: gentamicin; C: chloramphenicol; FT: nitrofurantoin; CT: colistin.

chicks placement. Another explanation is the selection of nalidixic acid- and/or enrofloxacin-resistant *E. coli* in parent animals (broiler breeders) and its vertical transmission in poultry production pyramid as suggested by others (Bortolaia et al., 2010; Petersen et al., 2006). Furthermore, pharmacokinetic considerations indicate that the use of fluoroquinolones against a nalidixic acid-resistant isolate may lead to the selection of isolates highly resistant to fluoroquinolones.

The study also revealed that a high level of resistance to tetracycline (92, 15%), trimethoprim-sulfamethoxazol (80, 39%) and amoxicillin (68, 62%). As compared to other studies in the same region (Hammoudi and Aggad, 2008; Aggad et al., 2010) and to those in other countries (Rahimi, 2013; Jing-Yu et al., 2013). The rate of antimicrobial resistance seems to know a progressive development for these antibiotics. In view of the whole range of antibiotics available in Algeria and the increasing and the lack of legislative restrictions on their use for therapy, prophylaxis, or growth promotion, the globally high incidence of antibiotic resistance observed in this study is not really surprising. In addition, in Algeria, treatments are usually given via the drinking water, and are always applied by the farmers themselves, after provision of the drugs by the veterinarian. Furthermore, precise dosing, an important aspect of the prudent use of antimicrobials, is often more difficult when applying mass medication. However, moderate level of resistance was observed for chloramphenicol (27, 45%) and nitrofurantoin (21, 56%). These results showed relatively lower resistance rates than those in other findings (Zahraei et al., 2006; Saberfar et al., 2008; Zakeri et al., 2012). These antibiotics are prohibited in veterinary medicine in Algeria. The fact of noticing a moderate resistance suggest an illicit use but however, less frequent. Low level of resistance was observed for gentamicin (7, 84%), and all the isolates were susceptible to colistin. Likewise, Aggad et al. (2010) reported a low rate of resistance to gentamicin (3%). In Algeria, gentamicin is actually used only in human medicine. In animals, colistin is used to prevent or treat infections caused by *E. coli* isolates. Recently, the emergence of plasmid-mediated colistin resistance involving the mcr-1 gene from bacteria was reported in many countries (Olaitan et al., 2016). In Algeria, colistin-resistant *E. coli* isolates with the associated mcr-1 gene were isolated from poultry. This is worrying because colistin is used as a last resort to treat multidrug-resistant pathogenic infections.

All the isolates examined in this study were resistant to at least three antibiotics (Figure 2). 98 (96, 07%) of the isolates were resistant to at least four antimicrobials and 93 (91, 17%) of the isolates were resistant to at least five antimicrobials. Three *E. coli* strains were resistant to all antibiotics tested, except colistin. A total of 28 antibiotic resistance patterns were distinguished. The most frequent are those designated in Table 1 as B, D, C and H. The most common multidrug resistant profile among these isolates was profile D (21, 56%), which was

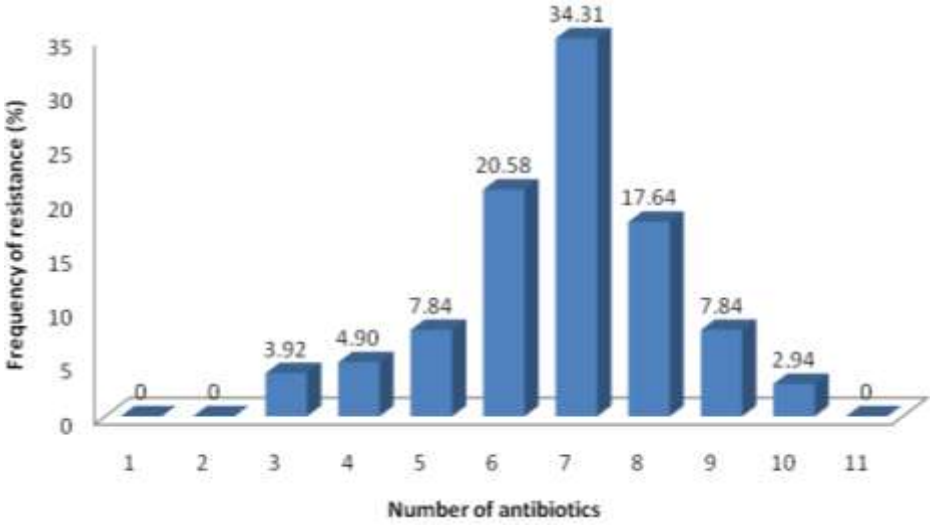

Figure 2. Prevalence of multiple antimicrobial resistance in avian *E. coli* isolates.

Table 1. The most frequent antibiotic resistance patterns in *E. coli* isolates (n=102).

Resistance patterns	Designation	Number of strains (%)
NA, UB, ENR, TE	A	4 (3, 92)
NA, UB, ENR, TE, SXT	B	6 (5, 88)
NA, UB, ENR, TE, SXT, N	C	4 (3, 92)
NA, UB, ENR, TE, SXT, AMX	D	22 (21, 56)
NA, UB, ENR, TE, SXT, AMX, N	E	4 (3, 92)
NA, UB, ENR, TE, SXT, AMX, CN	F	4 (3, 92)
NA, UB, ENR, TE, SXT, AMX, C	G	8 (7, 82)
NA, UB, ENR, TE, SXT, AMX, N, FT	H	6 (5, 88)
NA, UB, ENR, TE, SXT, AMX, N, C, FT	I	4 (3, 92)
NA, UB, ENR, TE, SXT, AMX, N, C, FT, CN	J	2 (1, 96)
NA, UB, ENR, TE, SXT, AMX, N, C	K	2 (1, 96)

NA: Nalidixic acid; UB: Flumequin; ENR: Enrofloxacin; TE: Tetracycline; SXT: Trimethoprrim-sulfamethoxazol; N: Neomycin; AMX: Amoxicillin; CN: Gentamicin; C: Chloramphenicol; FT : Nitrofurantoin.

resistant to nalidixic acid, flumequin, enrofloxacin, tetracycline, trimethoprim-sulfamethoxazol and amoxicillin. Chicken may receive different antimicrobial agents in a relatively short period of time, because of the fast growing cycle under field conditions. This may result in an undesireable combination of antimicrobials favouring an increase of antimicrobial resistance. The high prevalence of multidrug resistant *E. coli* in healthy broiler chickens (sampled a few days before slaughter) observed in this study is also observed by other findings. Dierikx et al. (2013) also observed a high prevalence of multiresistant *E. coli* in six-weeks-old broiler chickens. This can be explained on one hand by mass medication of whole flock including diseased animals, those assumed to be in the incubation period and those considered at risk of infection, and on the other hand by the short growing period of broiler chickens and the short withdrawal period after the cessation of treatment may not provide sufficient time for an adequate reduction of the resistant flora in the animals. Previous studies reported that resistant strains from the gut may contaminate poultry carcasses at slaughter and as a result poultry meats are often associated with multiresistant *E. coli* (Turtura et al., 1990). Further, a high rate of multiresistant in *E. coli* fecal isolates from poultry, poultry farmers and poultry slaughterers has been shown in the Netherlands (Van Den Bogaard et al., 2001). Spread of antibiotic resistance plasmids (De Been et al., 2014) or of antibiotic-resistant microorganisms from poultry to humans (Thorsteinsdottir et al., 2010) has been

reported. Of special, concern is the detection of high level of resistance to quinolone and fluoroquinolones in healthy broiler chickens. Indeed, fluoroquinolones also may be one of the only alternatives for the treatment of certain human infectious diseases. Furthermore, co-resistance of fluoroquinolones resistant *E. coli* to other antimicrobial agents, commonly used in human and veterinary medicine, such as trimethoprim-sulfamethoxazole and amoxicillin, is considered a serious health concern. Without an advisable use of antimicrobials, we may be faced with a public health crisis and return to the pre-antimicrobial era.

Conclusion

This study highlights the high prevalence of multidrug resistant *E. coli* among healthy broiler chickens in Western Algeria. These resistant bacteria may be transferred from food animals to humans by various means through the food supply and following contact with animals and their excreta, increasing the risk of treatment failure when this critical antimicrobial class is used in human patients. However, chicken meat represents the first animal protein source for the Algerian citizen; therefore, a tight surveillance of multiresistant bacteria from poultry products, should become a highest priority of the Algerian veterinarian authorities.

Conflict of interest

The authors have not declared any conflict of interest

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