



Antimicrobial Resistance Profile of Enteropathogens Isolated from Diarrhea Patients: Herbal Antimicrobials, a Ray of Hope

Bhoj R Singh*, Vinodh Kumar, Dharmendra K Sinha, Monika Bhardwaj, Archana Saraf and Prasanna Vadhana

Department of Epidemiology, ICAR-Indian Veterinary Research Institute, India

Abstract

Emergence of drug resistant microbes and their global spread is the biggest public health dilemma of the day. Enteropathogens are the biggest killers of neonates all over the globe. This study was conducted to understand antimicrobial drug resistance in bacteria causing enteric infections. A total of 199 bacterial strains isolated from faecal samples of diarrhoeic buffalo calves (8), foals (14), children (7), goat kids (7), piglets (74), chicks (9), pups (2) and cattle calves (78), belonging to 21 genera of enteropathogens were tested for their sensitivity to 8 herbal antimicrobials and 25 conventional antimicrobials. Of the tested strains 38.2%, 29.6%, and 12.1% strains were resistant to extended spectrum β -lactam drugs, carbapenems, and produced metallo- β -lactamases (MBL), respectively. Of the 24 strains positive for MBL, 22 were New Delhi metallo- β -lactamases (NDM) producers and two produced Verona integron encoded MBL (VIM). Both the VIM positive strains were *Shewanella* species and 6, 7, and 9 NDM producers strains belonged to *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Escherichia coli*, respectively. About 60% strains had multiple drug resistance (MDR) and 7.5% had multiple herbal antimicrobial resistant (MHAR). Among the herbal antimicrobials ajowan essential oil (AEO) was the most effective and inhibited all the strains, followed by cinnamaldehyde (98.2%), cinnamon essential oil (96%), holy basil essential oil (92.5%), carvacrol (91.9%), thyme essential oil (87.1%), *Zanthoxylum rhetsa* essential oil (21.3%) and patchouli essential oil (6.6%). Tigecycline was the most effective (*in vitro*) antibiotic on the strains tested inhibiting 83.7% strains followed by chloramphenicol (81.2%), moxalactam (81.1%), imipenem (78.2%), gentamicin (74%), and colistin (71.4%), other drugs could inhibit less than 70% of the strains. Erythromycin (1.3%) and ampicillin (17.1%) were the least effective antibiotics. Study revealed high levels of antimicrobial drug resistance in enteropathogens with a ray of hope with herbal antimicrobials.

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*Correspondence:

Bhoj R Singh, Department of Epidemiology, ICAR-Indian Veterinary Research Institute, 438-MLB, IVRI, Izatnagar, India,

E-mail: brs1762@yahoo.co.in

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Introduction

After pneumonia and respiratory tract infections diarrhea is the 2nd leading cause of mortality in children killing almost 760000 children every year [1-3] and treatment recommended include oral rehydration therapy with antibiotics [4,5]. On the other hand emerging drug resistance in bacteria specifically in enteropathogens is the imminent threat for survival of children [6]. The situation is not much different in animals and birds, diarrhea is the biggest killer of animal neonates [4,7-9] and antimicrobial drug resistance is the ever swelling problem in veterinary practice too [10]. The days of antibiotics are predicted to be countable and search for alternative therapies is felt all over the world [11]. Occurrence of herbal antimicrobial (HAR) and antibiotic drug resistance (ADR) has been reported in enteropathogens of birds [12], pigs and calves of cattle and buffaloes [13,14]. However, only little is known about comparative efficacy of the herbal antimicrobials resistance on clinical isolates of bacteria causing enteric infections. This study was conducted to evaluate the herbal as well as conventional antimicrobials for their activity on enteropathogens isolate from clinical cases of diarrhea.

Materials and Methods

Bacterial strains

A total of 199 bacterial strains isolated from faecal samples (Table 1) of referred clinical

Table 1: Herbal antimicrobial drug resistance (HADR) in bacteria isolated from cases of diarrhea.

Genus of Bacteria	Species of Bacteria (no. of isolates)	Source of isolation (No. of isolates)	Percent of strains sensitive to the herbal antimicrobials								
			ZREO	TEO	AEO	Carvacrol	PEO	CEO	HBEO	Cin-CHO	MHAR
Acinetobacter [3]	<i>Acinetobacter haemolyticus</i> (2), <i>A. lowffii</i> (1)	Piglets (2), calf (1)	33.3	NT	100.0	100.0	50.0	100.0	100.0	100.0	0.0
Aeromonas [2]	<i>Aeromonas bestiarum</i> (1), <i>A. salmonicida</i> (1)	Piglets (2)	50.0	NT	100.0	100.0	100.0	50.0	100.0	100.0	0.0
Alcaligenes [4]	<i>Alkaligenes denitrificans</i> (4)	Piglets (4)	0.0	NT	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Citrobacter [4]	<i>Citrobacter freundii</i> (4)	Foals (2), piglets (2)	100.0	NT	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Edwardsiella [6]	<i>Edwardsiella tarda</i> (5), <i>E. hoshiniae</i> (1)	Piglets (5), calf (1)	0.0	100.0	100.0	100.0	0.0	100.0	50.0	100.0	33.3
Enterobacter [16]	<i>Enterobacter agglomerans</i> (16)	Calves (5), foal (1), human (1), parrot (1), piglets (8)	7.1	87.5	100.0	100.0	0.0	86.7	100.0	100.0	6.3
Erwinia [3]	<i>Erwinia ananas</i> (1), <i>E. carotovora</i> (1), <i>E. tacheiphila</i> (1)	Calves (1), piglets (2)	50.0	100.0	NT	100.0	0.0	50.0	100.0	NT	0.0
Escherichia [101]	<i>E. coli</i> (98), <i>E. fergusonii</i> (2), <i>E. hermannii</i> (1)	Calves (49), buffalo calves (8), dog pups (2), foals (6), human (3), goat kids (5), birds (2), piglets (26)	13.5	89.8	100.0	100.0	9.4	98.7	93.7	97.26027	8.9
Haemophilus [1]	<i>Haemophilus</i> spp. (1)	Piglet (1)	0.0	NT	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Hafnia [1]	<i>Hafnia alvei</i> (1)	Calf (1)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Klebsiella [11]	<i>Klebsiella oxytoca</i> (1), <i>K. pneumoniae</i> ssp <i>pneumoniae</i> (10)	Calves (2), foals (2), human (2), birds (4), piglet (1)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Moraxella [1]	<i>Moraxella osloensis</i> (1)	Goat kid (1)	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0
Pasteurella [1]	<i>Pasteurella multocida</i> (1)	Duck (1)	0.0	NT	NT	100.0	0.0	NT	NT	NT	0.0
Pragia [1]	<i>Pragia fontium</i> (1)	Calf (1)	NT	NT	NT	100.0	0.0	NT	NT	NT	0.0
Proteus [12]	<i>Proteus mirabilis</i> (11), <i>P. penneri</i> (1)	Calves (3), emu (1), foals (2), piglets (6)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Providencia [1]	<i>Providencia alkalifaciens</i> (1)	Goat kid (1)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Pseudomonas [17]	<i>Pseudomonas aeruginosa</i> (17)	Calves (13), piglets (4)	76.5	0.0	NT	13.3	0.0	100.0	0.0	NT	11.8
Raoultella [3]	<i>Raoultella terrigena</i> (3)	Calves (1), foal (1), human (1)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Salmonella [8]	<i>Salmonella Typhimurium</i> (1), <i>S. Kentucky</i> (7)	Piglets (8)	50.0	NT	NT	N	0.0	NT	NT	NT	0.0
Serratia [1]	<i>Serratia marcescens</i> (1)	Piglet (1)	0.0	0.0	NT	100.0	0.0	100.0	100.0	NT	100.0
Shewanella spp. [2]	<i>Shewanella</i> spp. (2)	Piglets (2)	0.0	NT	100.0	100.0	0.0	100.0	100.0	100.0	0.0
Total (199)			21.3	87.1	100.0	91.9	6.6	96.0	92.5	98.2	7.5

cases of diarrhea in buffalo calves (8), foals (14), children (7), goat kids (7), piglets (74), chicks (9), pups (2) and cattle calves (78) and available in epidemiology laboratory as glycerol stocks were revived and were confirmed to the identity using biochemical and growth characteristics [15,16]. Bacterial isolates belonging to 21 genera of enteropathogens, dominated by members of *Enterobacteriaceae* (168) and *Pseudomonadaceae* (17) and few others were included in the study. All the strains were maintained on nutrient agar slants at 4°C throughout the study.

Antibiotic sensitivity assay

The bacterial isolates were tested *in vitro* for antibiotic sensitivity using disc diffusion method [17] on Mueller-Hinton (MH) agar (BD, India) plates to determine extended spectrum-β-lactam (ESBL), carbapenem antibiotic resistance and determination of metallo-β-lactamase activity [12]. Antiotic sensitivity assay was performed using discs (BD, BBL Sensidiscs) of ampicillin (10 µg), amoxicillin (30 µg), amoxicillin clavulanic acid (50+10 µg), aztreonam (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefalexin (10 µg), ceftriaxone (10 µg), cefotaxime (10 µg), cefotaxime clavulanic acid (10 µg),

chloramphenicol (25 µg), ciprofloxacin (10 µg), colistin (10 µg), cotrimoxazole (25 µg), erythromycin (15 µg), gentamicin (30 µg), imipenem (10 µg), meropenem (10 µg), moxalactam (10 µg), nalidixic acid (30 µg), nitrofurantopin (300 µg), piperacillin (100 µg), piperacillin tazobactam (100+10 µg), tetracycline (30 µg) and tigecycline (15 µg). All tests were repeated for conformity and diameters of zone of bacterial growth inhibition (ZI) around discs were measured in millimeter (mm). To determine metallo-β-lactamase activity double disc diffusion assay for key hole reaction was performed using meropenem/ imipenem discs along with ethylene diamine tetra acetic acid (EDTA) on MH agar as described earlier [17,18].

Herbal antimicrobial sensitivity of bacteria was determined against essential oils (EO) of ajowan (AEO), cinnamon (CEO), holy basil (HBEO), patchouli (PEO), thyme (TEO) and *Zanthoxylum rhetsa* seed coats (ZREO) procured from Nagaland Fragrance Ltd, Dimapur, and cinnamaldehyde (Cin-CHO) and carvacrol (Sigma) discs prepared to contain 1µL EO per disc [12,19] using disc diffusion assay on MH agar plates and ZI was measured in mm in the similar way as done for antibiotic discs [18].

Table 2: Different primers used in PCR for detection of carbapenemase genes.

S. No	Gene	Primers sequence	Cyclical condition	Product size (bp)	Reference
1	blaIMP	F- GGAATAGAGTGGCTTAAYTCTC R- GGTTTAAAYAAAACAACCAC	95°C x 5 m/95°C x1m – 55°C x1m – 72°C x1m (35 Cycles)/72°C x10 m	232	Poirel et al. [20]
2	blaVIM	F-GATGGTGTGGTTCGCATA R- CGAATGCGCAGCACCAG	95°C x 5 m/95°C x1m – 55°C x1m – 72°C x1m (35 Cycles)/72°C x10 m	390	Poirel et al. [20]
3	blaOXA	F-GCGTGGTTAAGGATGAACAC R- CATCAAGTTCAACCCAACCG	95°C x 5 m/95°C x1m – 55°C x1m – 72°C x1m (35 Cycles)/72°C x10 m	438	Poirel et al. [20]
4	blaNDM	F-GGTTTGGCGATCTGGTTTTTC R- CGGAATGGCTCATCACGATC	95°C x 5 m/95°C x1m – 55°C x1m – 72°C x1m (35 Cycles)/72°C x10 m	621	Poirel et al. [20]
5	blaKPC	F-CGTCTAGTTCTGCTGTCTTG R- CTTGTCATCCTGTGAGGCG	95°C x 5 m/95°C x1m – 55°C x1m – 72°C x1m (35 Cycles)/72°C x10 m	798	Poirel et al. [20]

Detection of carbapenemase producing genes and virulence factors by PCR

The genomic DNA and plasmid DNA was extracted by QIAamp DNA Mini Kit and QIAamp Mini prep Kit (Qiagen, India), respectively. The primers and amplification conditions for genotypic detection of carbapenemase genes are given in Table 2 [20]. Amplified positive PCR products were purified using gel purification kit (Qiagen, India) and cloned in p-Drive cloning vector (Qiagen, India). The plasmids containing the expected insert were sequenced by Sanger dideoxy method using commercial sequencing services (Eurofins Ltd, Bangalore). After sequencing, homology searches were made using BLAST algorithm available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi> to confirm the identity of the genes amplified.

Statistical analysis

Antibiotic sensitivity data of each of the strains was recorded on Excel sheet along with its source of isolation and analyzed for correlation among ZIs of different antimicrobials. To find association between different sources of bacteria and antibiotics sensitivity of different types of bacteria chi-square (χ^2) test statistics were used.

Results

A total of 199 bacterial strains isolated from faecal samples of diarrhoeic buffalo calves (8), foals (14), children (7), goat kids (7), piglets (74), chicks (9), pups (2) and cattle calves (78) revived from glycerol stocks available in Epidemiology Laboratory were and were confirmed to the identity using biochemical and growth characteristics. Bacterial isolates belonged to 21 genera of enteropathogens (Table 1), dominated by members of Enterobacteriaceae (168) and Pseudomonadaceae (17). Resistance to different herbal antimicrobials and conventional antimicrobials varied among strains of different genera (Table 2 and 3). In the study 38.2%, 29.6%, and 12.1% strains produced extended spectrum B-lactamases (ESBL), carbapenemases and metallo- β -lactamases (MBL), respectively (Table 4). The drug resistance was rampant and 60.3% strains were classified as multiple drug resistant (MDR) and 7.5% as multiple herbal antimicrobial resistant (MHAR). Of the 24 strains positive for MBL, 22 were New Delhi Metallo- β -Lactamase (NDM) positive and two produced Verona Integron encoded MBL (VIM). Both the VIM positive strains belonged to *Shewanella* species while of the 22 NDM producers, 6, 7, and 9 strains belonged to *P. mirabilis*, *P. aeruginosa* and *E. coli*, respectively. All NDM positive strains possessed the NDM gene on plasmid only.

Equally high number of strains of *E. coli* and *Pseudomonas* spp. were more often resistant to ZERO (Table 1) than strains of *Klebsiella*, *Proteus* and *Enterobacter* spp. (p, <0.01). However, TEO was as effective to inhibit growth of *E. coli* as of other members of Enterobacteriaceae (p, >0.1) but *Pseudomonads* were more commonly resistant to TEO than *Enterobacter* spp. (p, 0.02) and *Escherichia* spp.

strains (p, 0.0003). All *klebsiellae* and most of the *Proteus* strains were more often resistant to ampicillin, even more than strains of *Pseudomonads*. *Pseudomonads* were more commonly (p, <0.03) resistant to HBEO, cinnamaldehyde and carvacrol than strains of other bacteria except of *Proteus* spp. (p, 0.4).

About 21% bacteria were sensitive to ZREO (Table 1 and 4). Zone of bacterial growth inhibition around discs (ZI) of ZREO had no positive association with ZI of the other herbal antimicrobials or antibiotics tested in the study. However, significant (p, 0.01-0.05) negative association was evident among ZIs of ZREO and carvacrol (r, -0.48), nitrofurantoin (r, -0.39), chloramphenicol (r, -0.31), amoxicillin clavulanic acid (r, -0.42), tigecycline (r, -0.61), cefotaxime (r, -0.36), ceftaxime (r, -0.4) and aztreonam (r, -0.39).

More than 87% bacterial isolates were sensitive to TEO (Table 1) and ZI around TEO discs had significant (p, 0.01-0.05) positive correlation with ZI of strains to carvacrol (r, 0.56), and moxalactam (r, 0.42). The ZI induced by TEO rarely had negative correlation to sensitivity of bacteria to other antimicrobials except of erythromycin (r, -0.04), and holy basil essential oil (r, -0.2) but it was statistically insignificant.

Ajowan essential oil (AEO) was the most effective antimicrobial inhibiting all the 199 strains tested (Table 1 and 4) and ZI by AEO was positively correlated (p, 0.01) with the ZI induced by carvacrol (r, 0.51). Interestingly ZI by AEO had insignificant (p, >0.05) correlation with ZI caused by ZREO (r, 0.1), patchouli essential oil (r, 0.1), nitrofurantoin (0.27), imipenem (r, 0.13), amoxicillin (r, 0.02), and ceftaxime (r, 0.01). The ZI induced by AEO had an insignificant negative correlation (p, >0.05) with MDR (r, -0.11).

Most of the isolates of bacterial strains (91.9%) from diarrhoeic cases were sensitive to carvacrol. Zone of inhibition of bacteria by carvacrol was positively correlated (p, <0.05) with ZI caused by other herbal antimicrobials including TEO (r, 0.56), AEO (r, 0.51), CEO (r, 0.42) and cinnamaldehyde (r, 0.37). The positive correlation of ZI by carvacrol was also significant (p, <0.05) with ZIs of tetracycline (r, 0.37), nitrofurantoin (r, 0.59), chloramphenicol (r, 0.44), ceftazidime (r, 0.49), meropenem (r, 0.29), amoxicillin clavulanic acid (r, 0.41), tigecycline (r, 0.8), cefotaxime (r, 0.48), moxalactam (r, 0.31), ceftaxime (r, 0.32), cefepime (r, 0.3), aztreonam (r, 0.38) and piperacillin tazobactam. However, a significant (p, <0.01) negative correlation of carvacrol sensitivity was seen with sensitivity to ZREO (r, -0.48) and with MDR (r, -0.45).

Patchouli essential oil (PEO) was the least effective herbal antimicrobial in the study and it inhibited only 6.6% strains (Table 1 and 4). The ZI induced by PEO had no significant correlation to ZI around discs of any of the other herbal or antibiotic antimicrobial but had a significant (p, <0.05) negative relation with ZIs by moxalactam (r, -0.27), ceftaxime (r, -0.34), and piperacillin (r, -0.27).

Table 3: Antimicrobial drug resistance (ADR) in bacteria isolated from cases of diarrhea.

Genus of Bacteria	Percent of strains sensitive to the antimicrobials												Production of			MDR
	A	T	G	Nt	Na	CO	CF	C	Ca	CI	TG	AT	ESBL	CR	MBL	
<i>Acinetobacter</i> (3)	0.0	100.0	100.0	50.0	0.0	100.0	100.0	100.0	33.3	50.0	100.0	33.3	66.7	33.3	0	100.0
<i>Aeromonas</i> (2)	0.0	100.0	100.0	100.0	100.0	100.0	50.0	100.0	100.0	100.0	NT	50.0	50	50	0	50.0
<i>Alcaligenes</i> (4)	NT	100.0	100.0	25.0	100.0	100.0	75.0	100.0	100.0	100.0	100.0	25.0	75	100	0	75.0
<i>Citrobacter</i> (4)	0.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	50.0	0	0	0	0.0
<i>Edwardsiella</i> (6)	0.0	66.7	83.3	100.0	50.0	66.7	100.0	100.0	100.0	25.0	100.0	100.0	33.3	16.7	0	33.3
<i>Enterobacter</i> (16)	10.0	56.3	93.8	62.5	66.7	53.3	68.8	81.3	60.0	80.0	84.6	62.5	68.8	18.8	0	62.5
<i>Erwinia</i> (3)	33.3	66.7	100.0	66.7	33.3	50.0	100.0	100.0	33.3	100.0	100.0	50.0	0	33.3	0	100.0
<i>Escherichia</i> (101)	20.0	41.1	63.7	88.2	36.8	43.4	42.9	88.6	52.5	70.5	97.3	60.2	48.5	23.8	9.9	64.4
<i>Haemophilus</i> (1)	NT	100.0	100.0	NT	NT	NT	100.0	100.0	NT	NT	NT	100.0	0	100	0	0.0
<i>Hafnia</i> (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	100	0	0	100.0
<i>Klebsiella</i> (11)	0.0	36.4	90.9	45.5	66.7	63.6	63.6	100.0	100.0	100.0	100.0	100.0	27.3	0	0	63.6
<i>Moraxella</i> (1)	0.0	100.0	100.0	100.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0	0.0	0	0	0	100.0
<i>Pasteurella</i> (1)	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	NT	NT	NT	NT	0	0	0	0.0
<i>Pragia</i> (1)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	NT	NT	NT	0	0	0	0.0
<i>Proteus</i> (12)	40.0	16.7	66.7	28.6	20.0	50.0	83.3	66.7	70.0	11.1	50.0	50.0	8.3	50	41.7	41.7
<i>Providencia</i> (1)	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	100.0	100.0	100	0	0	100.0
<i>Pseudomonas</i> (17)	11.8	11.8	70.6	5.9	7.1	6.3	64.7	12.5	0.0	87.5	0.0	0.0	0	88.2	41.2	88.2
<i>Raoultella</i> (3)	0.0	66.7	100.0	66.7	100.0	66.7	66.7	100.0	100.0	100.0	100.0	50.0	66.7	0	0	33.3
<i>Salmonella</i> (8)	25.0	50.0	100.0	50.0	100.0	100.0	100.0	100.0	50.0	75.0	NT	NT	0	0	0	0.0
<i>Serratia</i> (1)	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.0	100.0	0.0	0	0	0	0.0
<i>Shewanella</i> spp. (2)	NT	100.0	0.0	NT	NT	NT	50.0	100.0	NT	NT	NT	0.0	0	100	100	100.0
Total (199)	17.1	45.9	74.0	68.5	41.3	48.1	57.4	81.2	54.9	71.4	83.7	53.8	38.2	29.6	12.1	60.3

Cinnamon essential oil (CEO) was effective on 96% isolates of bacteria tested (Table 1). Its ZI had significant ($p, <0.01$) correlation ($r, 0.66$) with ZI produced by cinnamaledehyde (Cin-CHO, active ingredient of cinnamon oil) and tigecycline ($r, 0.47$). The ZI by both CEO and Cin-CHO had a significant ($p, <0.05$) negative correlation with ZI induced by meropenem ($r, -0.024$) and imipenem ($r, -0.37$).

Holy basil essential oil inhibited growth of 92.5% bacteria but ZI by HBO was not is significant correlation with any of the herbal or other antimicrobials except a significant ($p, 0.05$) negative correlation with ZI induced by imipenem ($r, -0.32$). Resistance to three or more (multiple) herbal antimicrobials (MHAR) was detected in 7.5% strains tested and was in negative correlation to sensitivity of strains to ZREO ($r, -0.52$), TEO ($r, -0.64$), PEO ($r, -0.45$) and chloramphenicol ($r, -0.25$).

Tigecycline was the most effective (*in vitro*) antibiotic on bacterial strains associated with diarrhea inhibiting 83.7% strains followed by chloramphenicol (81.2%), moxalactam (81.1%), imipenem (78.2%), gentamicin (74%), and colistin (71.4%), other drugs could inhibit less than 70% of the strains only, erythromycin (1.3%) and ampicillin (17.1%) being the least effective ones (Table 3). Among the antibiotics, ZIs by erythromycin, colistin and tigecycline could not be correlated well to ZIs produced by most of the other antimicrobials. Exception was a positive correlation between ZI induced by erythromycin and ZIs by ampicillin, tetracycline and chloramphenicol. Correlation in ZIs of tigecycline was positive with ZIs induced by tetracycline ($r, 0.44$), nitrofurantoin ($r, 0.65$), cotrimoxazole ($r, 0.35$), chloramphenicol ($r, 0.53$), ceftazidime ($r, 0.41$), meropenem ($r, 0.32$), amoxicillin +

clavulanic acid ($r, 0.35$), cefotaxime ($r, 0.4$), aztreonam ($r, 0.37$) and piperacillin ($r, 0.33$). The correlation in antimicrobial activity (ZI) other antimicrobials was significantly ($p, \leq 0.05$) correlated with ZIs of each other. Multiple antimicrobial drug resistance (MDR) was evident in >60% isolates from diarrhea cases and was negatively correlated with ZIs by most of the antimicrobials but colistin. However, MDR had a little positive correlation with MHAR ($r, 0.21$) and a little ($p, >0.05$) negative correlation with ZIs induced by most of the herbal antimicrobials (except ZREO and PEO). The correlation of MDR was strongly negative ($p, <0.01$) with ZIs caused by carvacrol ($r, -0.45$).

In general pseudomonads were more often ($p, <0.05$) resistant than strains of other bacteria for most of the antibiotics (Table 3) including tetracycline, meropenem, imipenem, ceftazidime, cefoxitin, moxalactam, cotrimoxazole, chloramphenicol, tigecycline, cefotaxime, aztreonam, cefepime and piperacillin tazobactam. However, there was no significant difference in sensitivity of strains of different genera for piperacillin, ceftriaxone, ciprofloxacin, ampicillin, gentaycin and tetracycline. For tigecycline and gentamicin, there was no significant difference in sensitivity of the strains of most of the genera except the *E. coli* strains were often more resistant to the two drugs than *Enterobacter* strains ($p, <0.02$). Amoxycillin was equally less effective on most of the bacteria associated with diarrhea but *Proteus* strains were often more commonly amoxycillin resistant than klebsiellae ($p, 0.02$) and pseudomonads ($p, 0.01$).

Among Enterobacteriaceae members (except *Proteus* strains) capability of ESBL production was more common than among pseudomonads ($p, <0.02$). However, metallo- β -lactamase (MBL)

Table 4: Antimicrobial resistant (in %) bacteria isolated from different sources.

Antimicrobials	Buffalo calf	Cattle calf	Foals	Piglets	Human	Goat kids	Chicks	Pups	Total
Number of strains tested	8	78	14	74	7	7	9	2	199
ZREO	100.0	73.1	70.0	78.6	NT	100.0	100.0	100.0	78.7
Thyme essential oil	0.0	20.0	NT	18.8	0.0	0.0	0.0	NT	12.9
Ajowan essential oil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carvacrol	0.0	15.6	0.0	2.0	0.0	0.0	0.0	0.0	8.1
Patchouli essential oil	87.5	94.7	100.0	91.0	100.0	85.7	100.0	100.0	93.4
Cinnamon essential oil	0.0	0.0	0.0	11.1	0.0	0.0	0.0	0.0	4.0
Holy basil essential oil	12.5	1.9	0.0	12.8	16.7	0.0	0.0	50.0	7.5
Cinnamaldehyde	12.5	2.1	0.0	0.0	0.0	0.0	0.0	0.0	1.8
Ampicillin	75.0	85.5	33.3	79.5	85.7	100.0	88.9	100.0	82.9
Tetracycline	87.5	61.5	28.6	36.8	42.9	85.7	88.9	100.0	54.1
Gentamicin	25.0	40.5	14.3	15.8	14.3	0.0	11.1	100.0	26.0
Nitrofurantoin	12.5	36.5	38.5	25.9	28.6	14.3	55.6	0.0	31.5
Nalidixic acid	62.5	71.2	37.5	31.8	42.9	100.0	85.7	100.0	58.7
Cotrimoxazole	87.5	61.1	7.1	35.7	14.3	100.0	77.8	100.0	51.9
Ciprofloxacin	75.0	53.4	14.3	21.1	42.9	100.0	55.6	100.0	42.6
Chloramphenicol	25.0	27.4	0.0	5.8	14.3	57.1	0.0	50.0	18.8
Ceftazidime	37.5	59.2	0.0	33.3	28.6	14.3	66.7	100.0	45.1
Meropenem	0.0	44.4	0.0	44.4	16.7	0.0	33.3	50.0	37.7
Imipenem	0.0	20.0	0.0	35.4	14.3	0.0	0.0	50.0	21.8
Amoxicillin	87.5	80.6	42.9	62.7	85.7	85.7	100.0	100.0	73.2
Amoxicillin +clavulanic acid	37.5	52.5	28.6	48.8	16.7	28.6	66.7	100.0	47.1
Erythromycin	100.0	100.0	100.0	97.9	100.0	85.7	100.0	100.0	98.7
Colistin	0.0	27.4	66.7	37.3	0.0	14.3	12.5	100.0	28.6
Tigecycline	0.0	27.1	0.0	11.1	0.0	0.0	0.0	0.0	16.3
Cefotaxime	37.5	53.4	0.0	34.1	42.9	14.3	66.7	100.0	43.8
Cefotaxime+ clavulanic acid	25.0	50.0	0.0	100.0	NT	NT	0.0	100.0	50.0
Ceftriaxone	37.5	46.0	0.0	37.0	42.9	14.3	66.7	50.0	40.3
Moxalactam	0.0	17.0	0.0	31.4	0.0	0.0	33.3	50.0	18.9
Cefoxitin	0.0	52.1	50.0	61.9	42.9	71.4	33.3	100.0	52.5
Cefepime	0.0	57.8	100.0	29.0	16.7	14.3	0.0	100.0	39.4
Aztreonam	37.5	53.8	14.3	39.6	50.0	28.6	66.7	100.0	45.5
Piperacillin	62.5	67.4	0.0	52.9	83.3	71.4	66.7	100.0	63.2
Piperacillin + tazobactam	25.0	50.8	33.3	34.3	33.3	0.0	66.7	100.0	41.0
Extended spectrum β -lactam resistant	100.0	46.2	14.3	21.6	71.4	85.7	33.3	0.0	38.2
Carbapenem resistant	0.0	38.5	0.0	35.1	14.3	0.0	11.1	50.0	29.6
Metallo β -lactamase producers	0.0	11.5	0.0	18.9	0.0	0.0	0.0	50.0	12.1
Multiple drug resistant	100.0	75.6	28.6	37.8	71.4	100.0	88.9	50.0	60.3

production was more often associated with pseudomonads and *Proteus* strains than in strains of any other bacteria in the study (Table 3).

Comparison among strains of the same bacteria from different origin was not possible for most of the bacteria because of the few numbers of strains to compare (<10). However, when comparison was made among *E. coli* strains of piglet and of calf origin it was evident that piglet origin strains were more often resistant to cefotaxime clavulanic acid (p, 0.01), moxalactam (p, 0.03) and more

often producer of ESBL (p, 0.001) than those of calf origin, but not much difference was evidenced in their carbapenem sensitivity (p, 0.09).

Bacteria isolated from different animals and birds differed in sensitivity for many antimicrobials (Table 4). Comparatively more of the bacteria from diarrheic foals (p, <0.02) were sensitive to ZREO, ampicillin, tetracycline, cotrimoxazole, ciprofloxacin, amoxicillin, moxalactam, and aztreonam than strains of calf and piglet origin. In contrast, bacterial strains causing diarrhea in foals were more often

resistant to colistin and cefepime than strains associated with piglet and calf diarrhea. There was no significant ($p, >0.1$) difference among bacterial strains causing diarrhea in piglets and calf for sensitivity to most of the antimicrobials except that the piglet origin strains were more often sensitive to tetracycline ($p, <0.01$), nalidixic acid ($p, <0.01$), cotrimoxazole ($p, <0.01$), ciprofloxacin ($p, 0.002$), amoxicillin ($p, 0.003$) and cefepime ($p, 0.01$) than those associated with calf diarrhea. Although MBL producers were more common among strains associated with piglet diarrhea than those with foals and calf diarrhea, ESBL producers were significantly more common among strains associated with calf diarrhea than strains causing piglet ($p, 0.001$) or of foal ($p, 0.03$) diarrhea. All the 7 isolates from diarrhoeic kids and 8 from buffalo calves had MDR, followed by bacterial strains from chicks (88.9%), cattle calves (75.6%), children (71.4%), pups (50%), piglets (37.8%) and foals (28.6%).

Discussion

Diarrhea being one of the biggest killers of neonates is getting more and more difficult to be treated because of fast emergence of MDR strains of enteropathogens [1,6,14, 21,22]. Search for alternatives to antibiotics and alternative therapies for infections are the hottest topic globally [23,11]. Herbal antimicrobials are often considered as a feasible alternate since long [24,25] but emergence of resistance for herbal antimicrobials in recent years has jeopardized the hypothesis [11]. Therefore, this study comparing *in vitro* efficacy of herbal and conventional antimicrobials may advance our understanding of the alternative therapies of the future for emerging MDR strains.

In the study bacteria belonging to 21 genera were recorded in association of causing diarrhea, however members of *Enterobacteriaceae* and *Vibrionaceae* accounted for causation of the majority (~85%) of the cases. Similar observations have also been reported in earlier studies too [1,7]. Other 29 strains associated with diarrhea were belonged to 7 genera (*Acinetobacter*, *Alkaligenes*, *Haemophilus*, *Moraxella*, *Pasteurella*, *Pseudomonads* and *Shewanella*) and have rarely been associated with diarrhea [7]. The isolation of members other than of *Enterobacteriaceae* from clinical cases might be just the opportunists those populated the intestinal tract after antimicrobial drug therapy or might be having some potential to cause diarrhea and may be important for further studies.

The study revealed that AEO, TEO and carvacrol were effective against >87% strains, quite more than many of the antibiotics similar to earlier observations [12,19]. Both AEO and TEO has similar active component i.e., carvacrol, a thymol derivative [26], thus positive correlation observed among ZIs induced by all the three (AEO, TEO and carvacrol) was an expected observation. It might be due to similar mechanism of their antibacterial activity [26,27]. Though cinnamon essential oil and its active ingredient cinnamaldehide, acts in a bit different way to kill bacteria than carvacrol, had good correlation with antimicrobial activity of carvacrol [27,28]. In the Study, AEO was the most effective antimicrobial inhibiting all the strains tested, though reported to have carvacrol as an active antimicrobial agent in it [11], was quite more effective than carvacrol itself indicating either presence of some more potent antimicrobial) or synergy among the different components of AEO and needs further investigation.

Ajowan essential oil inhibited 100% strains but in earlier studies cinnamon essential and cinnamaldehyde were ranked the most active herbal antimicrobials followed by TEO [11,29], the difference might be to either due the testing of different kind of bacteria in the earlier

studies or AEO might be the really better one, large comparative studies can possibly reveal the truth. The least effective PEO and ZREO have rarely been tried against enteric bacteria thus comparison is not feasible. Another reason for non-inclusion of ajowan or AEO in the top 20 herbal antimicrobials of the world [30] might be due to less reported work on the herb and its occurrence in less research intensive regions.

All the 6 most effective herbal antimicrobials (AEO, TEO, carvacrol, CEO, HBEO and cinnamaldehide) had negative correlation with MDR i.e., more the drug resistance less were their sensitivity to the herbal drugs. The observation revealed that the popular concept that herbal drugs are more effective on MDR strains [28] does not appears to be true. Our observations are in concurrence to earlier observation reporting isolation of drug resistant isolates even from herbal drugs [31]. The observations in our study might be better representative of the field condition due to inclusion of large number of clinical strains in the study than in earlier studies, mostly on laboratory and reference strains. Although the less active herbal antimicrobials PEO (6.6%) and ZREO (21.3%) were slightly more active on MDR strains than on non MDR-strains as observed earlier [28], their utility as therapeutic agent appears to be low. The positive correlation in antibacterial activity of carvacrol and carvacrol containing essential oils (AEO, TEO) with ZI of most of antibiotics indicated that resistance to herbal antimicrobials (at least the more effective ones) goes hand in hand with antibiotic resistance and might be having the similar mechanism as proposed by [11]. It was also indicated by the positive correlation between MHAR and MDR ($r, 0.21$).

Presence of MDR in >60% strains of bacteria associated with clinical diarrhea was alarming and in concurrence to reports of shrinking antimicrobial activity against common bacteria [10,13,14,23,25]. Presence of MDR and ESBL producer strains of bacteria from veterinary clinical cases was expected as β -lactam antibiotics and their variants are commonly used and permitted in India and abroad in animals [4,16]. However, presence of carbapenem resistant VIM and NDM producing strains might be of serious public health concern as spread of pathogens by domestic and pet animals in environment is more common in India and other developing countries due to the prevailing animal husbandry practices permitting animals to soil almost all places, roads and households.

Ciprofloxacin and nalidixic acid were among the least effective antimicrobials inhibiting about 50% of the strains similar to β -lactam group of antibiotics. The observations points for the introspection of antibiotic use guidelines [32,33] as quinolones and fluoroquinolones are the most recommended and orally used drugs both in human and animals drugs for diarrhea cases [4,34] and the frequent use might be associated with common occurrence of resistance to these drug in enteric pathogens.

Detection of tigecycline, colistin and carbapenems resistance in 16.3%, 28.6% and 29.6% strains of bacteria associated with diarrhea in animals, respectively (Table 4), is again the confirmation of the fact that for newer antibiotics emergence of antimicrobial resistance (AMR) originates in humans and then spreads to different biotic and abiotic components of the environment [35]. Because due to one or other reasons most of the new antibiotic drugs are not used or used rarely in veterinary therapeutics. However, about spread of AMR strains there is consensus among scientists that several routes exist for spillover of AMR between the bacterial populations of humans

and animals in either direction [36].

In the study, 82.9%, 54.1% and 51.9% bacterial strains isolated from diarrhea cases were resistant to ampicillin, tetracycline and cotrimoxazole, respectively. The observations are in concurrence to earlier studies indicating common occurrence of resistance in bacteria of animal origin to tetracycline, penicillins and sulphonamides [35]. More than two decades ago majority of bacteria were reported to be resistant to ampicillin [37] and almost similar prevalence of resistance as observed in this study has been reported in other parts of India [14]. However, earlier studies [37] reported that resistance to tetracycline, penicillins and sulphonamides among chicken and swine bacterial isolates, and MDR was significantly higher in pig associated strains than those from cattle. In the present study strains associated with calf diarrhea were significantly more resistant to tetracycline ($p < 0.01$), nalidixic acid ($p < 0.01$), cotrimoxazole ($p < 0.01$), ciprofloxacin ($p < 0.002$), amoxicillin (0.003) and cefepime ($p < 0.01$) than strains causing pig or chick diarrhea. The contrasting results might be due to geographical difference and different husbandry practices in Northern India than in regions of earlier studies [35].

Chloramphenicol was one of the most effective antimicrobials in the study, though a very old drug, still very effective. The reasons for its effectiveness might be associated with not only prohibition of this drug use in animals but also with no or limited use of the antibiotic in humans nowadays. Otherwise, imipenem, meropenem, colistin and piperacillin are the antibiotics not used in animals due to non-permission and also due to economics, but resistance, to these new generation antibiotic drugs, is much more common in bacteria isolated from animals. The observation indicated that it is not necessary to use an antibiotic at a designated place to isolate the resistant pathogen for the very antibiotic, but the use of the antibiotic anywhere in the vicinity might be more important. Thus, nowadays, WHO and other global and national agencies are targeting to limit the overall antibiotic use rather than limiting the partial or segmental use of the antibiotics [3,12,32,33,38,39].

Multiple herbal antimicrobial drug resistance (MHAR) observed in the present study in 7.5% of the strains of bacteria causing diarrhea appears to be much lower than reported earlier in bacteria associated with foods of vegetable origin [40], enteric bacteria of house geckos [12] and bacteria causing other systemic infections in animals [40,19]. It might be due to difference in microbiome of different animals and systems or organs of the same animal and difference in enteric bacteria of different animals. Effect of source animal on MDR and MHAR observed in the study further proved the fact. However, the most important determinant for lower MHAR observed in the present study might be use of a bit different set of herbal antimicrobials to determine MHAR than used in earlier studies [12,19,21,40]. Therefore, for the judicious use of the term MHAR global consensus is need of the time to avoid the misuse of the term and to diminish the confusion in the literature. To implement the policy the Codex Alimentarius Code of Practice to Minimize and Contain Antimicrobial Resistance provides guidance on the responsible and prudent use of antimicrobials in food animals. In countries like India and in many other parts of the world integrated farming system is practiced where livestock, humans and the environment are intimately connected and microbe spillover from animals to man or vice versa might be responsible for fast spread of AMR [41]. Therefore, to contain the AMR the problem should be looked from a "One Health" perspective and an interdisciplinary approach needs to be practiced [42,43].

The study concludes that AMR should not be seen separately for herbal antimicrobials and antibiotics and both may go hand in hand. The study indicated that we cannot rely too much for long on herbal antimicrobials too. Though some of the herbal antimicrobials (AEO, TEO, CEO) are very effective *in vitro* to contain the growth of bacteria associated with diarrhea their use for therapeutics needs more studies towards standardization of their quality as drug, their side effects, and posology. The study also indicated that MDR strains are not extra sensitive to herbal antimicrobials rather they behave in the same way as for antibiotics specifically for their sensitivity to the most effective herbal antimicrobials.

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