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Antimicrobial Activity of Citronella Essential Oil on Antimicrobial Drug Resistant Bacteria from Veterinary Clinical Cases

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Abstract

Study on citronella essential oil (CEO) sensitivity of 217 microbial strains of 65 species, isolated from animals with different disease conditions, revealed that citronella oil inhibited growth of only 10.6% strains. CEO inhibited *Candida* but of no *Aspergillus* strain. CEO inhibited 22 of 211 bacterial strains. Ampicillin was the least effective antibiotic but inhibited 41.2% bacterial strains. Gram positive bacteria (GPBs) were 4.5 more sensitive (p, 0.0006) to CEO than Gram negative bacteria (GNBs). More GNB strains (p, 0.02) were multi-drug resistant (MDR) type than GPB strains. Probability of CEO resistant was high in MDR strains (p, 0.006). Most of the *Brucella abortus* strains had MDR (83.3%). Strains of swamp buffalo origin were more (p, 0.08) commonly CEO (96.6%) resistant than strains of dog (81.3%) origin. MDR was the maximum in abortion associated (51.2%) strains and minimum in diarrhoea associated strains (25%). The study indicated that CEO is not an effective antimicrobial against veterinary clinical isolates. Antimicrobial drug resistance and CEO resistance patterns of bacteria were dependent on type of pathogen, its source and association with disease in animals and may be important for deciding an effective antimicrobial therapy.

Keywords: Citronella oil; Antimicrobial; Antibacterial; MDR; *Brucella*; *Klebsiella*; *Salmonella*

Introduction

Citronella essential oil (CEO) is extracted from an herbaceous grass like tropical plant, *Cymbopogon nardus*, through steam distillation. Though it contains more than 22 compounds, geraniol, trans-citral, cis-citral, geranyl acetate, citronellal (6-octenal, 3, 7-dimethyl) and citronellol are the major constituents [1]. Citronellal alone constitutes about 29.6% of CEO [2]. Its use started long back as mosquito repellent [3]. CEO had immunomodulatory effect [4]. Besides, it has also been reported antifungal and antimicrobial [1,2,5-8]. It has been used as an alternative to commercial antibiotics in aquaculture [2] and also in aromatherapy for acne cures [9]. As an antimicrobial, citronella essential oil (CEO) has been shown to be inhibitory for about 50% strains of bacteria and fungi [7].

Citronella oil though effective against several species of bacteria is not equally active against all of them, minimum inhibitory concentration (MIC) varied significantly for different pathogens (0.12% to >2%, v/v), minimum for *Staphylococcus aureus* and *Candida albicans* and more for *Salmonella, Serratia* and *Pseudomonas* strains [6]. In another study [10] on *Propionibacterium acnes,* CEO had MIC equivalent to 0.125% (v/v) against all 5 strains tested. Recently [2], CEO has been reported inhibitory to 36 microbes of aquatic origin with an MIC ranging between 0.244 µg/ml for *Pseudomonas* and *Salmonella* to 0.977 µg/ml for *Edwardsiella tarda* and *Aeromonas* strains, which is much lower than earlier reports [6] for similar strains. CEO was found effective against *S. aureus, Bacillus subtilis, Proteus vulgaris* and *Pseudomonas aeruginosa* but could not inhibit *Escherichia coli* and *Klebsiella pneumoniae* strains and had MIC >12.8 mg/ ml [11]. The wide variation in MIC of CEO for different strains of bacteria of different as well as of the same species and of different origin necessitated re-examination of antimicrobial activity of CEO on bigger population of microbes. Therefore, this study was planned to examine the inhibitory effect of CEO on strains of wide variety of microbes of different origin using the same scale of testing. The study also attempted to establish association between conventional antimicrobial drug resistance and sensitivity to CEO. The study included 217 strains of microbes belonging to 65 species of 34 genera isolated from different disease conditions in animals and human beings.

Materials and Methods

Bacterial isolates

A total of 217 microbial strains including 211 bacterial strains, 5 *Aspergillus* and one *Candida albicans* isolates. Microbes in the study were either isolated from animals and their environment (211) or were reference (6) strains available in the laboratory were tested. All strains were revived and checked for purity on blood agar using standard microbiological methods [12,13]. Bacterial isolates (211) from more than 13 sources included in the study belonged to 62 species of 32 genera (Tables 1 and 2). Slants of pure cultures were stored on blood agar (BBL, Difco) until used for sensitivity assays.

Sensitivity to CEO

Six reference bacterial strains, 211 microbial cultures from different sources were tested in duplicate for their sensitivity to CEO (2 µL/disc) using disc diffusion method [14] on Mueller Hinton (MH) agar (BD BBL and Difco). All strains were tested for sensitivity on MH agar but *Moraxella, Streptococcus, Brucella* and *Pasteurella* strains were tested on brain heart infusion (BHI) agar (BD BBL and Difco) instead of MH agar due to their fastidious nature [15]. All strains were tested at 37°C under aerobic incubation, however, *Brucella* inoculated plates were incubated under 5% CO_2 enriched environment. The CEO discs were prepared to contain 2 µL of CEO in each disc as described earlier [14].

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Microbes (Number of strains tested)	Number of strains resistant to										
	CEO	T	G	Nf	Co	Cf	C	Az	Ct	A	MD
Acromobacter xylosoxidans (1)	1	0	0	0	0	0	0	0	0	0	0
Acinetobacter lwoffii (1)	1	0	1	1	1	0	0	1	1	1	1
Actinobacillus seminis (1)	0	0	0	0	1	0	0	0	0	0	0
Actinobacillus spp. (1)	1	1	1	1	1	1	1	1	1	1	1
Aerococcus spp. (2)	1	0	0	1	0	0	0	0	0	0	0
Aeromonas caviae (1)	1	0	0	0	0	0	0	0	0	1	0
Aeromonas eucranophila (1)	1	0	0	0	0	0	0	0	0	1	0
Aeromonas media (3)	2	1	0	0	1	0	1	0	0	3	1
Aeromonas salmonicida ssp. *salmonicida* (1)	1	0	0	0	0	0	0	0	0	0	0
Alkaligenes faecalis (2)	2	1	0	0	0	1	0	0	0	1	1
Alkaligenes denitrificans (1)	1	0	0	0	0	1	0	0	0	1	0
Aspergillus niger (1)	1	1	1	1	1	1	1	1	1	1	1
Aspergillums flavus (4)	4	4	4	4	4	4	4	4	4	4	4
Bacillus licheniformis (1)	1	0	0	0	1	0	0	0	0	0	0
Bacillus spp. (5)	3	0	0	0	1	0	0	0	1	1	0
Brucella abortus (12)	12	2	0	3	12	7	0	10	1	11	10
Candida albicans (1)	0	1	1	1	1	1	1	1	1	1	1
Citrobacter amalonaticus (1)	1	0	0	0	0	0	0	0	0	1	0
Citrobacter freundii (1)	1	0	0	0	0	0	0	0	1	0	0
Edwardsiella hoshniae (1)	1	0	1	0	0	0	0	1	0	1	1
Edwardsiella tarda (2)	2	1	0	0	1	0	0	0	0	2	1
Enterobacter agglomerans (20)	19	3	0	10	10	2	5	0	5	16	8
Enterobacter amnigenus bio group I (1)	1	0	0	0	0	0	0	0	0	1	0
Enterococcus spp. (6)	6	0	0	2	2	0	1	0	0	1	1
Erwinia ananas (2)	1	1	0	1	0	0	0	0	0	1	0
Erwinia cyperipedii (1)	1	1	0	1	1	1	0	1	0	1	1
Escherichia coli (45)	43	27	3	4	18	19	7	8	11	31	23
Hafnia alvei (1)	1	1	0	1	0	0	0	0	0	1	1
Klebsiella pneumoniae (16)	16	6	0	6	4	1	1	4	3	16	6
Kluyvera crocrescens (1)	1	0	0	0	0	0	0	0	0	0	0
Kluyvera ascorbata (1)	1	0	0	0	0	0	0	0	0	0	0
Listeria Monocytogenes (1)	0	0	0	0	0	0	0	0	0	0	0
Micrococcus spp. (2)	2	0	0	0	1	0	0	0	1	1	1
Moraxella osloensis (2)	1	0	0	0	1	0	0	0	0	0	0
Morganella morganii (1)	1	0	0	1	1	0	0	1	0	1	1
Pasteurella multocida B (2)	1	0	0	0	0	0	0	0	0	0	0
Pasteurella langaensis (1)	0	0	0	1	0	0	0	0	0	0	0
Pasteurella multocida D (1)	1	0	0	0	0	0	0	0	0	0	0
Pasteurella pneumotropica (2)	2	0	0	0	0	0	2	0	0	0	0
Pediococcus spp. (1)	1	0	0	0	0	0	0	0	0	0	0

Pragia fontium (2)	2	1	0	1	0	0	0	1	0	0	0
Proteus mirabilis (4)	4	4	0	2	4	1	1	3	4	2	4
Proteus penneri (2)	2	1	0	1	1	0	0	1	0	1	1
Pseudomonas aeruginosa (8)	8	3	1	5	5	1	3	2	4	6	5
Pseudomonas pseudoalcaligenes (2)	2	1	0	1	1	0	1	1	0	2	1
Pseudomonas testosteronii (2)	2	1	0	1	1	0	1	1	0	2	1
Pseudomonas vesicularis (1)	0	1	0	0	1	0	0	0	0	0	0
Raoultella terrigena (4)	4	1	1	2	3	1	0	2	1	4	2
Salmonella Kentucky (2)	2	2	0	0	0	2	0	0	0	2	2
Salmonella Adelaide (2)	2	0	0	0	0	0	0	0	0	0	0
Sphingomonas echinoides (2)	2	0	0	1	0	0	0	0	0	1	0
Staphylococcus aureus (8)	7	0	1	1	2	2	0	1	1	3	1
Staphylococcus carnosus (5)	5	1	5	2	4	5	3	4	4	5	5
Staphylococcus caseolyticus (2)	0	0	0	0	0	0	0	1	2	0	0
Staphylococcus delphini (1)	1	0	0	0	0	0	0	1	1	0	0
Staphylococcus epidermidis (2)	1	0	0	0	0	0	0	0	0	0	0
Staphylococcus gallinarum (1)	1	1	0	0	1	0	0	0	0	0	0
Staphylococcus intermedius (2)	2	1	0	1	1	0	0	0	0	0	1
Staphylococcus lentus (1)	0	0	0	0	1	0	0	0	0	0	0
Staphylococcus sciuri (1)	1	0	0	0	1	0	0	0	0	0	0
Streptococcus equi ssp. *zooepidemicus* (8)	7	3	2	2	6	4	2	5	3	1	4
Streptococcus macacae (1)	0	0	0	0	0	0	0	0	0	0	0
Streptococcus porcinus (1)	0	0	0	0	0	0	0	0	0	0	0
Streptococcus suis (1)	1	1	0	0	0	1	0	1	0	0	1
Vibrio cholerae Non O1 (1)	1	0	0	0	0	0	0	0	0	0	0
Total (217)	194	73	22	59	95	56	35	57	51	129	92
*Percent resistant bacteria (211)	89.6	31.8	7.6	25.1	42.2	23.7	13.7	24.2	21.3	58.8	37.9
Resistant GPBs (52)	40	45	44	43	30	40	46	39	39	40	14
Resistant GNBs (159)	149	99	151	115	92	121	136	121	127	47	72

*Calculated only for bacterial strains (n=211); GPBs, Gram positive bacteria; GNBs, Gram negative bacteria; number in parentheses indicate the number of strains tested; A, ampicillin (10 µg); AZ, azithromycin (15 µg); CEO, citronella essential oil (2 µL); Ct, Cefotaxime (30 µg); C, chloramphenicol (30 µg); Cf, ciprofloxacin (5 µg); Co, cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.25 µg)); G, gentamicin (10 µg); MD, multiple drug resistance (resistant to 3 or more antimicrobials); Nf, nitrofurantoin (300 µg); T, tetracycline (30 µg).

Table 1: Antimicrobial drug and citronella essential oil (2 µL disc) resistance in strains of veterinary clinical significance and isolated from different sources.

Source (nos. of strains)	**Types of Bacteria tested (number of isolates)**	**Number of strains resistant to**										
		CEO	T	G	Nf	Co	Cf	C	Az	Ct	**A**	MD
Air (3)	*Pseudomonas aeruginosa* 1, *Actinobaccilus seminis* 1, *Staph. gallinarum* 1	2	1	0	1	3	0	0	0	1	1	1
Buffalo (6)	*Alkaligenes faecalis* 1, *Alk. Denitrificans* 1, *Erwinia cyperipedii* 1, *Klebsiella pneumoniae* 2, *Pse. testosteronii* 1	6	4	0	3	3	4	2	2	0	6	4
Cattle(53)	*Aerococcus* spp. 2, *Aeromonas eucranophila* 1, *Bacillus* spp. 1, *Brucella abortus* 10, *Enterobacter agglomerans* 2, *Ent. Amnigenus* 1, *Enterococcus* spp. 2, *Erwinia ananas* 1, *Escherichia coli* 15, *K. pneumoniae* 6, *Morganella morganii* 1, *Pasteurella langaensis* 1, *Pediococcus* spp. 1, *Pragia fontium* 2, *Proteus mirabilis* 1, *Proteus penneri* 1, *Pse. Aeruginosa* 1, *Staph. aureus* 2, *Streptococcus equi* ssp. *zooepidemicus* 2	48	19	2	13	24	14	2	17	9	38	24
Dog (16)	*E. coli* 1, *P. mirabilis*, 3, *P. penneri* 1, *Pse. Aeruginosa* 1, *Raoultella terrigena* 1, *Sphingomonas echinoides* 2, *Staph. Caseolyticus* 2, *Staph. Delphini* 1, *Staph. Intermedius* 2, *Staph. Lentus* 1, *Staph. sciuri* 1	13	7	1	6	9	2	0	7	7	4	7
Elephant (7)	*Bacillus* ssp. 1, *E. coli* 1, *Ent. Agglomerans* 1, *Enterococcus* spp. 1, Micrococcus spp. 1, *Staph. Aureus* 1, *Strept. equi* ssp. *zooepidemicus* 1	7	0	0	0	2	0	0	0	3	2	1

Horse (9)	*Bacillus licheniformis* 1, *E. coli* 3, *R. terrigena* 1, *Strept. zooepidemicus* 4	9	6	3	3	9	5	2	4	4	3	6
Human (20)	*Acinetobacter* lwoffii 1, *Actinobacillis* spp. 1, *Bacillus* spp. 1, *E. coli* 1, *Moraxella osloensis* 2, *Pasteurella pneumotropica* 2, *Pse. Aeruginosa* 1, *Edwardsiella hoshniae* 1, *R. terrigena* 2, *Staph. Aureus* 1, *Staph. Carnosus* 5, *Staph. Epidermidis* 1, *Strept. zooepidemicus* 1	17	3	9	5	10	9	7	9	8	13	10
Mithun (18)	*Citrobacter freundii* 1, *Ent. Agglomerans* 3, *Enterococcus* spp. 3, *Erwinia ananas* 1, *E. coli* 5, *Hafnia alvei* 1, *K. pneumoniae* 2, *Pse. Aeruginosa* 1, *Staph. aureus* 2	17	3	1	5	5	3	2	2	4	11	6
Peafowl (5)	*E. coli* 1, *Ent. Agglomerans* 2, *Staph. aureus* 2,	5	1	0	1	4	1	0	0	2	1	1
Pig (29)	*Achromobacter xyloxidans* 1, *Aeromonas media* 1, *Aeromonas salmonicida* ssp. *salmonicida* 1, *Edwardsiella. Tarda* 2, *E. coli* 12, *K. pneumoniae* 1, *Micrococcus* spp. 1, *P. multocida* type D 1, *Pse. Aeruginosa* 2, *Pse. Vesicularis* 1, *Salmonella* Adelaide 2, *Salmonella* Kentucky 2, *Strept. porcinus* 1, *Vibrio cholerae* Non O1 1	25	17	0	4	6	8	8	0	1	18	12
Rabbit(4)	*Aeromonas caviae* 1, *Bacillus* spp. 2, *Pse. aeruginosa* 1	4	0	0	1	1	0	1	1	1	3	1
Rhino (6)	*E. coli* 1, *K. pneumioniae* 1, *Pse. Pseudoalcaligenes* 1, *Strept. Macacae* 1, *Strept. Suis* 1, *Staph. aureus* 1	4	2	0	1	1	2	0	4	0	2	2
Swamp Buffalo (29)	*Aeromonas media* 2, *Alkaligenes faecalis* 1, *Citrobacter amalonaticus* 1, *Ent. Agglomerans* 12, *E. coli* 5, *K. pneumoniae* 4, *Kluyvera ascorbata* 1, *Kluy. Crocrescens* 1, *Pes. Pseudoalkaligenes* 1, *Pse. testosteronii* 1	28	4	0	10	10	1	5	3	5	20	9
Reference (6)	*Brucella abortus* 2 (Strain 19 and Strain 99), *Listeria Monocytogenes* 1 (MTCC839), *Pasteurella multocida* type B 2 (P52, Soron), *Staph. epidermidis* 1 (MTCC449)	4	0	0	0	2	1	0	2	0	2	2
Total		189	67	16	53	89	50	29	51	45	124	86

Number in parentheses indicate the number of strains tested; A, ampicillin (10 µg); AZ, azithromycin (15 µg); CO, citronella essential oil (2 µL); Ct, cefotaxime (30 µg); C, chloramphenicol (30 µg); Cf, ciprofloxacin (5 µg); Co, cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.25 µg)); G, gentamicin (10 µg); MD, multiple drug resistance (resistant to 3 or more antimicrobials); Nf, nitrofurantoin (300 µg); T, tetracycline (30 µg).
MTCC strains were procured from MTCC, Chandigarh, India, *Brucella* strains were procured from National *Brucella* Centre, and *Pasteurella* strains from CADRAD, Indian Veterinary Research Institute, Izatnagar, India.

Table 2: Source wise distribution of different bacteria (211) tested for sensitivity to antimicrobial drugs and citronella essential oil.

Citronella oil (CAS: 8000-29-1, Pcode 101178470) having geraniol as main constituent was purchased from Sigma Aldrich Chemie GmbH, Germany.

Antibiotic sensitivity assay

Sensitivity of all 217 strains including *Candida* and *Aspergillus* strains to antibiotics (ampicillin, 10 µg; azithromycin, 15 µg; cefotaxime, 30 µg; chloramphenicol, 30 µg; ciprofloxacin, 5 µg; cotrimoxazole, sulfamethoxazole 23.75 µg+trimethoprim 1.25 µg; gentamicin, 10 µg; nitrofurantoin, 300 µg and tetracycline, 30 µg) was determined with standard disc diffusion assay as per CLSI [16] guidelines on MH agar. Antibiotic sensitivity assay was performed in duplicate on Mueller Hinton (MH) agar (BD BBL and Difco). For *Moraxella, Streptococcus, Brucella* and *Pasteurella* strains instead of MH agar brain heart infusion (BHI) agar (BD BBL and Difco) was used [15]. All strains were tested at 37°C under aerobic growth conditions but *Brucella* strains were tested under micro aerobic environment in 5% CO_2 enriched environment in McInntosh jar. Results were interpreted as per CLSI [16] guidelines and strains were designated as sensitive or resistant depending on zone of inhibition. Strains resistant to three or more antimicrobials were classified as multiple drug resistant (MDR).

Statistical analysis

To determine correlation between diameter of zone of inhibition (in mm) of bacteria around antimicrobial and CEO discs correlation coefficient (r) was calculated using Microsoft Exel-7. To estimate association between sensitivity of bacteria to antimicrobials, including CEO and source of their source of isolation, species and association with disease, χ^2 test and odds ratio analysis was performed in MS Office Excel-2007. The statistical comparison was done for only those groups of bacteria or sources of bacteria where number (n) of strains tested was ≥ 10.

Results and Discussion

Results of antimicrobial and CEO sensitivity assays of 217 microbial strains of 65 species belonging to 34 genera (Table 1) revealed that citronella oil could inhibit growth of only 23 (10.6%) microbes. In the study none of *Candida* and *Aspergillus* strain was sensitive to any of the nine antimicrobials tested. Resistance of yeasts and molds to antibacterial drugs is constitutive and anticipated being inherited [15-17]. Only *Candida* but none of the five *Aspergillus* strain was sensitive to CEO. Although CEO has been reported earlier to inhibit growth of several fungi including reference *A. niger* [1,5,7,8,18] strains. However, it was not found inhibitory to any of the five potentially toxigenic *Aspergillus* strains in the present study. In earlier studies [2,6,10], CEO could inhibit growth of *A. niger* in concentrations higher than 0.125% (v/v) however in our experiment no inhibition was seen around discs containing 2 µL of CEO. This variation might be attributed due to several reasons including difference in strains, methodology and CEO itself as observed earlier [2,6,10] and also due to variation in composition of CEO [1].

Citronella oil could inhibit growth of only 22 (10.4%) out of 211 bacteria while the least effective antibiotic (ampicillin) could inhibit growth of 41.2% bacterial strains. Similar pattern of resistance in bacterial strains of different animal and environmental origin has been reported for other essential oils including closely related lemongrass oil [19].

Gentamicin was the most effective antimicrobial inhibiting 92.4% bacterial isolates followed by chloramphenicol (86.3%), cefotaxime (78.7%), ciprofloxacin (76.3%), azithromycin (75.6%), nitrofurantoin (74.9%), tetracycline (68.2%) and cotrimoxazole (57.8%). The resistance pattern for different antibiotics varied among strains of different species (Table 1) and strains of different sources (Table 2). Similar variation in sensitivity of bacterial strains to various antimicrobials is of common occurrence all over the world [16] and similar patterns have been reported earlier for bacterial isolates from veterinary clinical cases in India too [20].

Sensitivity to CEO was more common among GPBs with 4.5 times higher odds of being sensitive than GNBs (p, 0.0006) similar to their

higher sensitivity to gentamicin (p, 0.01) than GNBs. Similar pattern of higher resistance in GNBs has also been reported with lemongrass oil, a close relative of citronella oil [19].

Probability of GPBs being resistant for tetracycline (p, 0.001) and ampicillin (p, <0.0001) was significantly higher than GNBs and similar pattern has been observed for these antibiotics earlier worldwide [16,17,20]. GNBs were significantly more MDR type than GPBs (p, 0.02). MDR for 3-5 drugs was significantly more common in GNBs (p, 0.001) but there was no much difference in MDR for six or more drugs between GPBs and GNBs (p, 0.36). Higher proportion of MDR strains among GNBs is common all over the globe and has been commonly reported in India and abroad [17,20-22].

Significantly (p, 0.006) less number of MDR strains was sensitive to CEO, than non-MDR strains (Table 3; Figure 1). Similarly, probability of ampicillin resistance was more in CEO resistant strains. However, no significant association could be established between resistance to CEO and other antimicrobials used in the study. Only few studies have earlier compared the sensitivity of MDR and non-MDR strains to herbal oils or herbal antimicrobials, however, on meta-analysis of published data [23] it was evident that bacteria having MDR were more commonly resistant to essential oil of lemongrass and sandal wood. In the same study [23] no correlation could be found between ampicillin resistance and resistance to essential oil of sandal wood, lemongrass, *Artemesia vulgaris*, and patchouli. However, sensitivity to alcoholic extract of *Zanthxylum rhetsa* and *Ageratum conyzoides* was significantly (p, 0.02) associated positively with sensitivity to ampicillin [23].

Though bacterial strains of 62 species belonging to 32 genera were included in the study, number of strains belonging to different species were <10 except of *Brucella abortus* (12), *Enterobacter agglomerans* (20), *E. coli* (45), *Klebsiella pneumoniae* (16), *Pseudomonas* spp. (13), *Staphylococcus* spp. (23) and *Streptococcus* spp. (11). Statistical analysis for association of drug resistance with different bacteria was calculated for those bacteria with >10 strains included in the study.

On comparing sensitivity of strains belonging to different groups of pathogens (Table 1) it was evident that probability of CEO resistance in strains of *E. coli* (p, 0.02), *Klebsiella* (p, 0.03), *Brucella* (p, 0.05) and *Enterobacter* (p, 0.08) was much more than in strains of *Streptococcus* (72.7%). More strains of klebsiellae (p, 0.05) and brucellae (p, 0.08) were resistant to CEO than of staphylococci (78.3%) but there was no difference for CEO resistance among other types of bacteria. The similar variation in sensitivity of different bacteria to CEO has also been observed earlier [2].

All strains of klebsiellae, raoultellae, *staph. Carnosus*, salmonellae, and aeromonads (except a strain of *Aeromonas salmonicida* ssp. *salmonicida)* were resistant to ampicillin. Besides majority of brucellae (11/12), *E. agglomerans* (16/20), *P. aeruginosa* (10/13) and *E. coli* (31/45) were resistant to ampicillin. Ampicillin resistance in klebsiellae, raoultellae and aeromonads is common and often inherited, and ampicillin sensitive strains are rarely been reported [24,25]. Due to

MDR	**G+ve bacteria (source of isolation)**	**G-ve bacteria (source of isolation)**
9	*S. carnosus*1 (doctors' hand)	*Actinobacillus* spp. 1(UTI), *Ps. aeruginosa* 1 (UTI)
7	*S. carnosus* 2 (doctors' hand)	*E. coli* 3 (cattle aborted fetus 2, dog wound 1)
	*St. equi*ssp. *zooepidemicus*2 (horse nose)	*R. terrigena* 1 (horse nose)
		Proteus mirabilis 1 (dog otorrhea)
6	*S. carnosus* 1 (doctors' hand)	*Erwinia cyperipedii* 1 (buffalo aborted fetus)
	S. aureus 1 (infertile mithun)	*E. coli* 2 (cattle aborted fetus)
	St. equi ssp. *zooepidemicus* 1 (horse nose)	*B. abortus* 2, cattle aborted fetus
5		*E. coli* 6 (cattle aborted fetus 1, dead rhino 1, dead peafowl 1, dead pig 3)
		Brucella abortus 1 (cattle aborted fetus)
		Ent. agglomerans 2 (vagina of infertile mithun and swamp buffalo)
		K. pneumoniae 2 (vagina of infertile mithun 1, buffalo aborted fetus 1)
		Ps. pseudoalcaligenes 1 (vagina of infertile mithun)
		R. terrigena 1 (dog otorrhoea)
4	*S. carnosus*1 (doctors'hand)	*Aeromonas media* 1 (dead pig)
		B. abortus 4 (cattle aborted fetus 3, reference 1)
		Ent. agglomerans 4 (vagina of infertile mithun)
		E. coli 6 (dead pigs 3, human UTI 1, horse nose 1, cattle aborted fetus 1)
		K. pneumoniae 1 (vagina of infertile mithun)
		Morganella morganii 1 (cattle mastitis)
		Proteus penneri 1 (dog otorrhoea)
		Proteus mirabilis 2 (*dog otorrhoea)*
		Ps. aeruginosa 1 (dairy farm air)

3	*St. suis* 1 (dead rhino)	*Alkaligenes faecalis* 1 (buffalo aborted fetus)
	St.equi ssp. *zooepidemicus* 1 (calf diarrhoea)	*B. abortus* 3 (cattle aborted fetus2, reference 1)
	Enterococcus spp. 1 (cattle aborted fetus)	*Edwardsiella hoshniae* 1 (human UTI)
	S. intermedius 1 (dog wound)	*Edwardsiella tarda* 1 (pig diarrhoea)
	Micrococcus spp. 1 (tooth canal of elephant)	*Ent. agglomerans* 2 (vagina of infertile mithun and healthy swamp buffalo)
		E. coli 6 (horse nose 1, cattle aborted fetus5)
		Hafnia alvei 1, infertile mithun vagina
		K. pneumoniae 3 (cattle mastitis 1, vagina of healthy swamp buffalo 1, pig nose 1)
		Ps.aeruginosa 1 (vagina ofinfertile mithun)
		Salmonella Kentucky 2 (spleen of dead pig)

B., Brucella; E., Escherichia; Ent., Enterobacter; K., Klebsiella; Ps., Pseudomonas; R., Raoultella; S. Staphylococcus; St., Streptococcus.; MDR, multiple drug resistance to number of antimicrobials (≥ 3). None of the MDR strains, except two *E. coli* from dead pigs (resistant to 5 drugs) and one *Strept. equi* ssp. *zooepidemicus* (resistant to three drugs) from diarrhoeic calf was sensitive to citronella essential oil.

Table 3: Multiple drug resistant (MDR) strains of Gram+ve and Gram-ve bacteria isolated from different sources.

frequent resistance to it, this antibiotic has never been considered an option for treatment of brucellosis and *Pseudomonas* infections [21,26].

With respect to tetracycline resistance, *E. coli* (p, <0.01) strains had more probability of being resistant (60%) to it than brucellae (16.7%), and enterobacteria (15%). Both *E. coli* (p, 0.0002) and *Klebsiella* (p, 0.08) strains were more often resistant to tetracycline than staphylococci (13%). Pseudomonads were more often resistant (46.2%) to tetracycline than *Enterobacter* (p, 0.05) and *Staphylococcus* ssp. (p, 0.03) strains. Though tetracycline is a comparatively older antibiotic and resistance to it is common in most of the bacteria [19], it (as doxicycline) is still considered drug of choice for treatment of brucellosis [26] due to sensitivity of brucellae to tetracyclines. In this study too brucellae were more often sensitive to tetracycline despite of having MDR.

For gentamicin, sensitivity of strains of different bacteria (73.9%-100%) didn't differ much, except for the strains of staphylococci and streptococci, which were more often resistant to gentamicin than strains of enterobacteria (p, 0.01, 0.05) and klebsiellae (p, 0.03, 0.08). Besides, *Staphylococcus* strains were also more commonly resistant to gentamicin (26.1%) than *E. coli* (p, 0.03) and *B. abortus* (p, 0.05). Aminoglycoside resistance in most of the GPBs including staphylococci and streptococci is reported more commonly than in GNBs[27-30]. Due to wide spectrum of activity and its effectiveness to inhibit majority of bacterial strain it is still considered to be the drug of last resort in many life threatening infections [27].

More number of *E. agglomerans* (50%) and *Pseudomonas* (53.8%) strains were nitrofurantoin resistant than strains of *Staphylococcus* (p, 0.03, 0.02) and *Streptococcus* (p, 0.08, 0.07) species. However, *E. coli* strains were less often resistant to nitrofurantoin (8.9%) than *Pseudomonas* (p, 0.0003) and *K. pneumoniae* (p, 0.008) strains. Similar pattern has been observed earlier for bacteria of veterinary clinical origin [15,20].

All *B. abortus* strains were resistant to co-trimoxazole and differed significantly from other bacteria (p, 0.02) and observations are in concurrence to earlier reports [26]. Probability of co-trimoxazole resistance was more among *Pseudomonas* (61.5%) strains than in *K. pneumoniae* (25%; p, 0.05) strains. In general, streptococci (45.5%; p, 0.02) and staphylococci (30.4%; p, 0.07) were more often resistant to ciprofloxacin than *K. pneumoniae* (6.3%), *Pseudomonas* (7.7%; p, 0.03), and *E. agglomerans* (10%; p, 0.02) strains. Similar to cocci, more *E. coli* (42.2%) strains were ciprofloxacin resistant than *K. pneumoniae* (p, 0.0009), *Pseudomonas* (p, 0.02) and *E. agglomerans* strains (p, 0.01). However, *B. abortus* were even more (58.3%) commonly resistant to ciprofloxacin than *E. agglomerans* (p, 0.003), *K. pneumoniae* (p, 0.003) and *Pseudomonas* (p, 0.007) strains. An observation of more ciprofloxacin resistance in cocci than among GNBs is in concurrence to earlier reports [16,17].

Resistance to chloramphenicol was not detected in brucellae but was common among pseudomonads (38.5%). However, sensitivity to chloramphenicol not varied significant (p >0.05) among strains of other bacteria (6.3-25%). Among pseudomonads chloramphenicol resistance was significantly more common than in strains of *B. abortus* (p, 0.02), *E. coli* (p, 0.07), *K. pneumoniae* (p, 0.03) and staphylococci (p, 0.08). Sensitivity of *Brucella* to chloramphenicol is a well-documented [26]. In recent years emergence of chloramphenicol resistance in pseudomonads has become a serious problem both in strains of human and animal origin [15,16].

More than 83% of the *B. abortus* strains were resistant to azithromycin while all the *E. agglomerans* strains were sensitive, all other bacteria stood in between the two extremes. *Brucella abortus* were more resistant to azithromycin than *E. agglomerans* (p, <0.0001), *E. coli* (p, <0.0001), *Pseudomonas* (p, 0.008) and *Staphylococcus* (p, 0.003) strains. More number of streptococci (54.5%) was resistant to azithromycin than *E. coli* (p, 0.01) but difference was insignificant with respect to strains of all other bacteria except all sensitive strains of *E. agglomerans*. Although azithromycin is broad spectrum antibiotic, it is recommended commonly for infection with GPBs [15,16]. Emergence of azithromycin resistance in GPBs from veterinary clinical cases is of importance having knowledge that this drug is rarely used in animals in India [28]. This might be due to horizontal transfer of either resistance genes (R-factors) or the bacteria itself from human to animals.

Sensitivity to cefotaxime among strains of different bacteria (8.3-30.8%) had no significant variation. However for ampicillin, streptococci (90.9%) and staphylococci (65.2%) were more sensitive than GNBs (<25%). Probability of being sensitive to cefotaxime was even more among streptococci than staphylococci (p, 0.05). More sensitivity to cefotaxime in GPBs than GNBs has also been reported earlier in bacteria of animal origin [20,29].

Most of the *B. abortus* strains (83.3%) were MDR type and MDR was much more common in brucellae than in other bacteria including

K. pneumoniae (p, 0.02), *E. agglomerans* (p, 0.03), *Pseudomonas* (p, 0.02), *Staphylococcus* (p, 0.02) and *Streptococcus* (p, 0.01) strains. Next to *B. abortus,* MDR was common in *E. coli* (51.1%) and it was significantly more common than in *E. agglomerans* (p, 0.03), *K. pneumoniae* (p, 0.03) and *Staphylococcus* (p, 0.004) strains. MDR in *Brucella* strains is rarely reported (Corbel, 2006) and its detection is of public health concern due to zoonotic nature of the pathogen. MDR strains of *E. coli* have haunted globally and are one of the common global problems [15,16,22].

Analysis of data for source of bacterial strains and antimicrobial drug resistance (Table 2) indicated that CEO resistance (96.6%) was relatively more common (p, 0.08) among strains isolated from swamp buffaloes (Figure 2) than in strains from dogs (81.3%). However, strains from other sources had no significant difference for sensitivity to CEO. The dogs and swamp buffaloes are two extremes in our range of sources of bacteria studied, one the closest to human and other roaming in semi-wild stage in Nagaland eating different types of herbs including citronella grass common in Nagaland [29,30]. The continuous exposure of microbes of swamp buffaloes to citronella might be responsible for harbouring CEO resistant strains by swamp buffaloes, though it needs further confirmation either it is their feeding habit or any other determinant involved.

Tetracycline resistance (TR) was most common in bacteria isolated from pigs (58.6%) and dogs (43.8%). TR was significantly less common in strains of human origin (15%) than strains of cattle (p, 0.08), dog (p, 0.06) and pig (p, 0.002) origin. More of the swamp buffalo origin bacteria were tetracycline sensitive (13.8%) than strains from cattle (p, 0.03), dogs (p, 0.03) and pigs (0.0004). Similarly more isolates of mithun origin (16.7%) were sensitive to tetracycline than isolates of dog (p, 0.08) and pig (p, 0.005) origin. Similar pattern for tetracycline sensitivity among bacteria of mithun and swamp buffalo origin has been observed in earlier studies too [29,31]. Higher resistance to tetracycline in bacteria of pig and dog origin might be due to consumption of more concentrate feed and also due to their similar food habits as of human and sharing of similar kind of microbe [15,17,20].

In contrast to tetracycline resistance, gentamicin resistance was significantly more common (p, <0.01) among bacteria of human origin (45%) than isolates from animals. Few strains of dog (6.3%), mithun (5.6%) and cattle (3.8%), had resistance to gentamicin but none of the isolates of swamp buffalo and pig origin been resistant to gentamicin. The restricted use of gentamicin in animals in India [28] might be considered responsible for low prevalence of gentamicin resistant bacteria in animals.

Most bacterial strains of pig origin (86.2%) were sensitive to nitrofurantoin while more (p, 0.07) strains isolated from dogs (62.5%) and swamp buffaloes (65.5%) were resistant. Nitrofurantoin resistance among bacterial strains from other animals had no significant variation (p, >0.2) in their sensitivity to nitrofurantoin. Nitrofurantoin is a rarely used drug in animals except limited use of similar drug furazolidone in wound dressing and in passaries in India [28] that is why despite of being an old antimicrobial resistance was comparatively uncommon.

Majority of strains of dog origin were resistant to cotrimoxazole (56.3%) while about 80% of bacterial isolates from pig were sensitive. Cotrimoxazole sensitivity among isolates from pigs was significantly lower than those from dogs (p, 0.02) and humans (p, 0.03) while there was not much difference among strains of other animals (p, >0.09). Cotrimoxazole though commonly used in animals [28] is not a preferred drug for pigs [15]. Less use of co-trimoxazole in pigs might be associated with occurrence of co-trimoxazole sensitive strains in pigs.

Sensitivity to ciprofloxacin was common in bacteria from swamp buffaloes (96.6%), mithuns (83.3%) and dogs (87.5%) while only 73.6% of cattle and 72.4% of pig origin strains were sensitive. However, only 55% strains of human origin were sensitive to ciprofloxacin which was significantly higher than for strains of dog (p, 0.04), mithun (p, 0.06) and swamp buffalo (p, 0.004) origin. Among animal origin strains, less isolates of swamp buffalo origin were resistant to ciprofloxacin than isolates of pig (p, 0.01) and cattle (p. 0.01) origin. Ciprofloxacin is one of the most widely used injectable antibiotics in human while its equivalent (enerfloxacin) in animals in India [15]. However, enerofloxacin has rarely been used in swamp buffaloes and mithuns because of their semi-wild nature and difficulty in use of injectable antibiotics [29,31]. Hence, occurrence of ciprofloxacin resistant strains in mithun and swamp buffaloes might be rarer than in other animals and humans.

None of bacterial isolates from dogs and only few from cattle (3.8%) and mithun (11.1%) had resistance to chloramphenicol while more (p, <0.1) strains of human (35%) and pig (27.6%) origin were resistant to chloramphenicol. Rare occurrence of chloramphenicol resistance among strains of animal origin might be due to less use of chloramphenicol for animals in India [15]. On the other hand, azithromycin resistance was rare in isolates of pig origin (0%) and common in strains of dog (43.8%) and human (45%) origin. The sensitivity to azithromycin was more common among strains of swamp buffalo (p, 0.03), mithun (p, 0.08) and pigs (p, 0.0006) than strains of cattle (p, <0.08), human (p, <0.03) and dogs (p, <0.03). Azithromycin is not used in animals [15] but is a commonly used antibiotic in humans and is available without prescription in India [32]. Occurrence of azithromycin resistance in bacterial strains of dog and human origin might be due to close cohabitation of both and sharing (exchange) of zoonotic bacteria among the two species [33].

Bacterial isolates of dog (43.8%) and human (40%) origin were more commonly resistant (p, ≤ 0.04) to cefotaxime than isolates from pig (3.4%), cattle (17%), swamp buffalo (17.2%) and mithun (22.2%). Sensitivity ratio among bacteria from pig was significantly higher (p, ≤ 0.07) than isolates of any other animal. Higher resistance to cefotaxime in dog and human origin isolates might be due to sharing/ exchange of bacteria with each other and more frequent use of cefotaxime both in human and dogs than in other animals in India [15,20].

Significantly more (p, ≤ 0.03) bacterial isolates from dogs (75%) were sensitive to ampicillin in comparison to other animals (Figure 2). Though very commonly used antibiotic in India, is rarely used in dog [15]; less use of the drug in dogs might be associated with frequent sensitivity of bacterial isolates of dog origin to ampicillin.

There was no significant difference (p, >0.18) with respect to MDR in bacterial isolates of different animal and human sources (Table 3). The observation is quite in contradiction to earlier observation on more frequent MDR in bacterial strains in human beings in India [34]. It might be due to fast spread of antimicrobial drug resistance and fast emergence and spread of MDR strains of bacteria both in animal and humans [17,27].

Analysis of drug resistance among bacterial isolates associated with different disease conditions (Figure 3) indicated that in general more of the bacterial strains isolated from diarrhoeic cases were sensitive to antimicrobials than those isolated in association of other disorders in animals and humans. It might be due to the fact that diarrhoea is often a multi-etiologic disorder and associated with disturbed microflora rather than specific pathogen establishing itself through acquisition of specific virulence and antimicrobial drug resistance [35]. However,

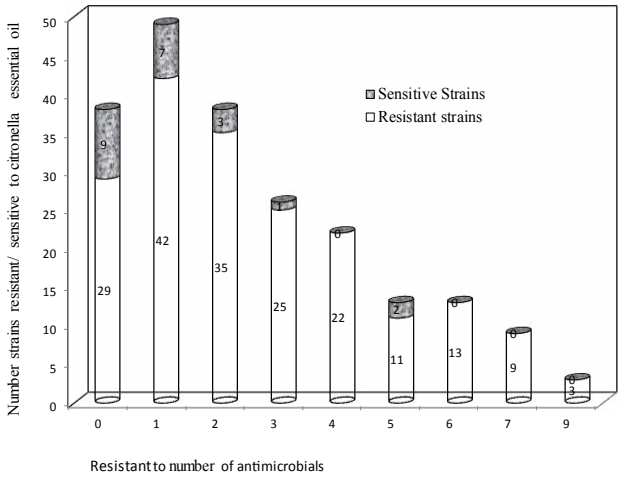

Figure 1: Multiple drug resistance and sensitivity to citronella essential oil in bacterial strains isolated from different animal sources.

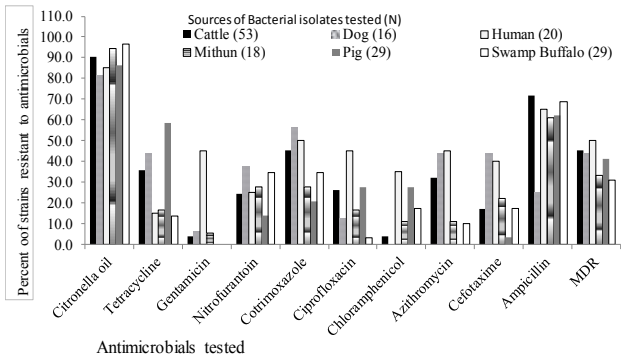

Figure 2: Antimicrobial drug resistance and multiple drug resistance (MDR) in bacterial strains from different sources.

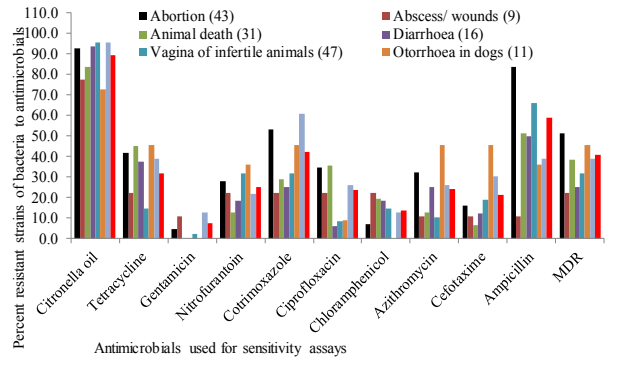

Figure 3: Antimicrobial drug resistance and multiple drug resistance (MDR) in bacterial strains associated with different disease conditions.

bacterial isolates from otorrhoea cases (27.3%) were more commonly sensitive to CEO than those associated with abortion (p, 0.06), infertility (p, 0.01) and respiratory tract infection (p, 0.05) cases. Majority of the bacteria isolated (95.7%) from infertility cases and respiratory tract infections were resistant to CEO. In contrast, significantly more (p, ≤ 0.05) bacteria isolated from infertility cases (85.1%) were sensitive to tetracycline than those associated with other ailments. It might be due to the fact that tetracycline is one of the rarely used drugs to treat uterine infections [36]. Gentamicin being one of the most effective antibiotics in the study could inhibit >95% bacteria associated with different disease conditions in animals and human beings. However gentamicin could inhibit only 87% of bacterial strains associated with respiratory tract infections (RTI). Nitrofurantoin was the least effective antimicrobial on bacteria isolated from otorrhoea (63.6%) and most effective on bacteria isolated from cases of septicaemia (87.1%) leading to mortality. Cotrimoxazole inhibited 75% of bacteria from diarrhoeic cases while only 39.1% bacteria isolated from RTI cases (p, 0.03) and 46.5% from abortion cases (p, 0.03). Bacteria from abortion (53.5%) and RTI (60.9%) cases (p, ≤ 0.05) were more commonly resistant to cotrimoxazole than those from diarrhoea (25%), infertility (31.9%) and mortality (29%) cases. Indiscriminate use of cotrimoxazole as non-prescription drug in animals in India [15] might be responsible for frequent occurrence of cotrimoxazole resistance. Ciprofloxacin inhibited >90% bacterial isolates from diarrhoea (93.7%), infertility (91.5%) and otorrhoea (90.9%) cases while it was significantly (p, ≤ 0.05) less effective on bacteria associated with RTI (73.9%), mortality (64.5%) and abortion (65.1%). It might be due to association of specific drug resistant pathogens with RTI and abortions, and also with frequent indiscriminate use of enerofloxacin in animals. Enerofloxacin has even been used as preservative in injectable vaccines intended for veterinary use in India [37,38]. None of bacterial isolates from otorrhoea and only 7% of isolates from abortions cases were resistant to chloramphenicol but >80% bacterial isolates associated with other ailments too were sensitive to chloramphenicol. About 90% bacteria associated with infertility and mortality were sensitive to azithromycin which was significantly (p, ≤ 0.05) higher than those from cases of otorrhoea (54.5%) and abortions (67.4%). Cefotaxime was the most effective on bacteria isolated from blood of dead (93.5%) and faeces of diarrhoeic (87.5%) animals; however, it was significantly less effective (p, ≤ 0.06) on bacteria causing otorrhoea (54.5%). Bacteria from abortion cases (83.7%) were more often resistant to ampicillin than isolates from blood of dead (p, 0.003), faeces of diarrhoeic (p, 0.008), and swabs from infertile (p, 0.05), otorrhoeic (p, 0.001) and RTI (p, 0.0002) cases. Bacteria isolated from otorrhoea (p, 0.07) and RTI (p, 0.03) were less resistant type even less than those from infertile animals.

The study indicated that some of the antimicrobials should be used with proper diagnosis and considering the system affected by the infection for effective antimicrobial therapy as indicated earlier [15,16,23]. With respect to MDR bacterial strains isolated from different disease conditions did not differ much (p, >0.05) however, proportion was maximum among strains isolated from abortion (51.2%) and minimum among strains associated with diarrhoea (25%). It indicated that the pathogens associated with abortion needs to be considered serious threat for future planning for MDR control. High level of MDR in abortion associated pathogens might be of immense public health concern because such pathogens may contaminate the environment at large scale during abortion in large animals. Moreover, citronella essential oil may not be very promising antimicrobial in veterinary use due to frequent resistance in bacteria of animal origin to it.

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