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Research Article

Antimicrobial Susceptibility Profile of Bacterial Culturome of Heart Blood Samples of Big Cats Died in Zoos and Wildlife Sanctuaries in Northern India

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Abstract

In India, little is understood precisely about the cause of death in big cats in zoos, wild and wildlife sanctuaries. The present study reports the bacterial culturome (culturable bacteria) of heart blood of leopards (5), lions (9) and tigers (26) found dead in zoos (30) or wildlife sanctuaries (10). From the samples submitted to Clinical Epidemiological study 145 bacterial (46 Gram positive and 99 Gram negative) strains (the group of isolates with a separate identity) belonging to 44 species (19 of G+ve and 25 of G-ve bacteria) were identified. From 17 (12 tiger, 1 leopard, 4 lions) heart blood samples bacteria of the single species were isolated in pure culture indicating cases of septicemia. The most common isolation as single bacteria type was of *E. coli* (5), followed by isolation of *Alcaligenes denitrificans* (2), *A. feacalis* (2), and one each of *Acinetobacter calcoaceticus, Bacillus cereus, Paenibacillus macerans, Enterobacter (Pantoea) agglomerans, Pseudomonas aeruginosa, Staphylococcus epidermidis, S. intermedius* and *Streptococcus pneumoniae*. Multiple drug-resistance (MDR) was detected in 73.1% and isolates belonged to 134 resistotypes. There was no significant (p, >0.05) difference in occurrence of herbal antimicrobial resistance of strains isolated from different animal of different locality. Significantly high probability (p, ≤0.04) of MDR strains and strains resistant to citral, tetracycline, nitrofurantoin, chloramphenicol and imipenem was recorded in samples from animals died in wildlife sanctuaries than those died in zoos. Of the 40 carbapenem resistant (CR) isolates identified from 16 (40.0%) heart blood samples, 21 (all Gram positive) were negative for MBL and 19 CR strains producing MBL belonged to 8 species of G-ve bacteria. The MIC of imipenem for MBL producer CR isolates ranged between 2 to 32 μg mL^{-1} while for those not produced MBL MIC ranged from 1.5 to 256 μg mL^{-1}, all the carbapenem susceptible isolates had imipenem MIC between 0.001 to 1.0 μg mL^{-1}. The study concluded multiplicity of bacteria in heart blood of big cats died in zoo and wildlife sanctuaries. Presence of multiple bacterial types in 57.5% samples suggests need for aseptic and timely collection of blood samples to understand the true etiology of fatality among big cats. Detection of MDR, ESBL and CR bacteria from 25%, 37.5% and 40% samples is alarming because of chances of spreading of AMR in the environment from animals suffering from infections with MDR strains and died in wild.

Keywords: Lion; Tiger; Leopard; Carbapenem-resistance; Herbal Drug-resistance; Blood Culture; MDR

Introduction

Death of big cats including tiger, lion, jaguar, leopard, and snow leopard in wild often goes un-noticed but in zoos, and protected sanctuaries can be monitored and investigated for the cause. Big cats being carnivores may acquire infection from their prey, contaminated water bodies and from each other. Big cats are often reported to suffer from a variety of parasitic (trichinosis,

filariasis, sarcoptic mange, pentastomiasis, echinococcosis, taeniasis, hepatozoonosis, babesiosis, theilerisis, tick infestation), bacterial (anthrax, tuberculosis, pasteurellosis, mycoplasmosis, anaplasmosis, colibacillosis, botulism, bartonellosis, actinomycosis, listeriosis, klebsiellosis, streptococcal pneumonia, morganelliasis), viral (rabies, canine distemper, feline immunodeficiency virus disease, feline Calicivirus disease, feline leukemia and panleukemia), and mycotic (ringworms, cryptococcosis) infections which may or may not be zoonotic [1-7].

Death due to bacterial causes is often associated with sepsis and can be confirmed through isolation of bacteria from heart blood and other vital organs. Isolation of bacteria from heart blood is indication of systemic infection causing bacteremia which may lead to septicemia (sepsis) and death. Sepsis, a systemic inflammatory response syndrome (SIRS), secondary to an infectious process in cats, is often present as diagnostic problem, the common signs are pyrexia (> 103.5°F), tachycardia (>225 bpm), increased respiratory rate (>40 per min), disturbed white blood cell count (>20, 000 or <5000 per µL) and early and transient hyperglycemia, followed by hypoglycemia (Costello, 2015). Most of the zoo and wild animals in India are reported to dye due to one or other kind of physical injuries, among the infectious disease in zoo or wild animals' septicemia and pneumonia are of rare occurrence and reported to be the cause of only 2.3% and 1.8% of the total deaths, respectively much behind the tuberculosis killing almost 5.8% of the zoo and wild animals [8]. In a study in India, besides septicemia, urinary tract infection, pleurisy, pneumonia, tuberculosis, pyometra, endometritis, enteritis, gastritis, gastric ulcers, gastroenteritis, hepatitis and peritonitis have been reported as important causes of mortality in tigers kept in zoos [9]. However, identity of infectious causes has rarely been attempted. This study was undertaken for revealing the diversity of bacteria isolated and identified from heart blood samples during postmortem of leopards, lions and tigers died in zoos and sanctuaries in Northern India.

Materials and Methods

Zoo/ wildlife veterinarians through Centre for wildlife (ICAR-IVRI, Izatnagar) submitted heart blood samples from dead animals aseptically collected during postmortem examination in sterile containers, transported on ice. All the samples were received in the Centre for Wildlife and forwarded for bacteriological analysis in Clinical Epidemiology Laboratory (ICAR-IVRI, Izatnagar). In the last 10 years, a total of 5 leopards', 9 lions' and 26 tigers' heart blood were examined through conventional blood culture method for aerobically and micro-aerobically growing bacteria [10]. For isolation of bacteria from samples, 100 ml of thioglycolate medium (BBL-Difco, USA) was inoculated with 1 mL of the blood sample and incubated for 24 h at 37°C. The growth from the thioglycolate medium was streaked onto 5% sheep defibrinated blood agar (BBL, Diffco, USA) plates in duplicate. One set was incubated at 37°C for 24 h-48h aerobically, and another micro-aerobically. Thereafter culture plates were observed every 24 h for isolated colonies. If no growth was observed on plates, thioglycolate broth was further incubated for 5 more days and then streaked as above. Three to five colonies of each different type were picked up and re-streaked onto blood agar plates and incubated at 37°C for 24 h for purification. The pure cultures were tested for morphological, culture, staining and biochemical characteristics using standard protocols [11,12]. Thereafter, bacterial isolates were classified up to genus and species level using criteria laid in Bergey's Manual of Determinative Bacteriology [13]. Those isolates could not be identified through conventional culture methods were confirmed with MALDI-ToF MS performed with a MALDI Biotyper Sirius system (Bruker Daltonics) for the identity.

All bacterial isolates were tested for antimicrobial susceptibility on Mueller Hinton agar plates (CLSI, 2015) through disc diffusion assay. In the study antibiotic discs (BBL-Difco, USA) of ampicillin (10 µg), amoxicillin+ clavulanic acid (50+10 µg), amoxicillin (30 µg), amoxicillin+ sulbactam (30+15 µg), azithromycin (15 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (30 µg), chloramphenicol (25 µg), ciprofloxacin (10 µg), colistin (10 µg), cotrimoxazole (25 µg), erythromycin 15 µg), gentamicin (30 µg), imipenem (10 µg), linezolid (30 µg), meropenem (10 µg), nitrofurantoin (300 µg), penicillin G (10 IU), piperacillin (100 µg), piperacillin + tazobactam (100+10 µg), tetracycline (30 µg), tigecycline (15 µg), and vancomycin (30 µg) were used. Isolates were classified into susceptible or resistant on the basis of diameter of zone of growth inhibition around antibiotic discs using criteria (wheresoever's available) laid down by CLSI [14,15]. The Gram-negative bacteria were not tested for vancomycin and linezolid susceptibility while Gram-positive bacteria were not tested for colistin and aztreonam susceptibility. Extended-spectrum β-lactamase (ESBL) and Metallo-β-lactamase (MBL) production was assured using E-test (BioMerieux, India Ltd.) and double disc

diffusion assays [14,15]. The minimum inhibitory concentration (MIC) of all bacteria resistant to carbapenems was determined by E-test (BioMerieux) for imipenem [14,15]. All bacterial isolates were also tested against herbal antimicrobials viz., ajowan (*Tachyspermum ammi*) seed oil, carvacrol, cinnamaldehyde, cinnamon (*Cinnamomum zeylanicum*) bark oil, citral, holy basil (*Ocimum sanctum*) oil, lemongrass (*Cymbopogon citrates*) oil, and thyme (*Thymus vulgaris*) oil procured from Sigma Aldrich (USA), and Naga Fragrance Ltd, Dimapur using the disc diffusion assay [16], each disc contained 1µL of the test herbal substance. Bacteria resistant to three or more classes of antibiotics or herbal antimicrobials were classified as multiple-drug-resistant (MDR) and multiple herbal drug-resistant (MHDR), respectively.

Results

Identification profile of bacteria from 40 samples of dead large cats revealed multiplicity of bacteria in postmortem heart blood (Table 1). From 40 samples, 145 strains of bacteria (46 Gram positive and 99 Gram negative) belonging to 44 species (19 of G+ve and 25 of G-ve bacteria) were identified (Table 2). From 12 of the 26 samples of tiger heart blood bacteria were isolated in pure culture while in other 14 (46.15%) cases more than one type of bacteria was detected. Similarly, of the 5 heart blood samples of dead leopards only one (20%) had single type of bacteria and others had two or more types of bacteria. From heart blood of 9 dead lions, 4 had only one type of bacteria each while 5 (55.56%) samples had multiple types of bacteria in the samples.

Case number	Habitat	Bacteria detected in heart blood of 26 dead tigers (number of resistotypes), from 12 samples only one type of bacteria
11	Sanctuary	*Enterobacter* (*Pantoea*) *agglomerans*
15	Sanctuary	*Aeromonas bestiarum, Citrobacter freundii, Escherichia coli*
48	Sanctuary	*Staphylococcus intermedius*
80	Sanctuary	*Streptococcus pneumoniae*
83	Sanctuary	*Aeromonas salmonicida, Enterobacter* (*Pantoea*) *agglomerans, Proteus mirabilis* (2)
89	Sanctuary	*Escherichia coli, Proteus vulgaris*
169	Sanctuary	*Enterococcus faecalis* (2), *Enterococcus faecium* (2)
41	Zoo	*Escherichia coli* (1)
49	Zoo	*Proteus mirabilis, Streptococcus milleri* (2)
54	Zoo	*Alcaligenes denitrificans, Bacillus subtilis* (2)
55	Zoo	*Escherichia coli* (3), *Proteus mirabilis* (2)
56	Zoo	*Acinetobacter calcoaceticus*
57	Zoo	*Alcaligenes denitrificans* (2)
58	Zoo	*Aeromonas salmonicida* ssp. *salmonicida* (2), *Streptococcus milleri*
59	Zoo	*Staphylococcus aureus, Streptococcus pyogenes*
88	Zoo	*Staphylococcus chromogenes, Staphylococcus lentus*
90	Zoo	*Escherichia coli*
100	Zoo	*Paenibacillus macerans*

103	Zoo	*Alcaligenes denitrificans, Enterococcus faecalis, Escherichia coli* (5), *Proteus mirabilis, Proteus vulgaris, Staphylococcus haemolyticus, Streptococcus milleri*
105	Zoo	*Alcaligenes denitrificans* (3), *Escherichia coli, Klebsiella pneumoniae* ssp. *pneumoniae* (2), *Proteus mirabilis, Staphylococcus aureus*
115	Zoo	*Alcaligenes faecalis, Enterobacter* (*Pantoea*) *agglomerans, Enterococcus faecalis* (3), *Enterococcus faecium, Staphylococcus aureus, Staphylococcus lentus* (2)
117	Zoo	*Pseudomonas aeruginosa* (2)
125	Zoo	*Escherichia coli* (4)
142	Zoo	*Alcaligenes faecalis* (2)
202	Zoo	*Enterococcus faecalis, Enterococcus faecium, Escherichia coli* (3), *Escherichia fergusonii*
205	Zoo	*Escherichia coli* (2)
Bacteria detected in heart blood of 5 dead leopards (number of resistotypes), from 1 sample only one type of bacteria		
71	Zoo	*Aeromonas popoffii, Escherichia coli, Moraxella osloensis, Moraxella phenylpyruvica* (3), *Proteus mirabilis* (2), *Staphylococcoccus arlettae, Streptococcus porcinus*
82	Sanctuary	*Aeromonas hyderophila, Pragia fontium*
123	Zoo	*Aeromonas bestiarum, Aeromonas trota* (2), *Enterobacter* (*Pantoea*) *agglomerans* (1), *Pseudomonas aeruginosa*
137	Zoo	*Staphylococcus epidermidis* (2)
169	Sanctuary	*Aeromonas trota* (2), *Enterococcus faecalis* (2), *Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae ssp. Pneumonia, Staphylococcus intermedius*
Bacteria detected in heart blood of 9 dead lions (number of resistotypes), from 4 samples only one type of bacteria		
98	Sanctuary	*Raoultella terrigena, Proteus mirabilis* (2)
114	Sanctuary	*Bacillus cereus*
61	Zoo	*Alcaligenes denitrificans*
73	Zoo	*Alcaligenes faecalis* (2)
80	Zoo	*Escherichia coli* (2), *Klebsiella oxytoca, Staphylococcus arlettae, Staphylococcus capitis* ssp. *capitis, Streptococcus pneumoniae* (2), *Streptococcus pyogenes*
114	Zoo	*Bacillus pantothenticus, Achromobacter xyloxidans, Aeromonas salmonicida ssp. salmonicida*
121	Zoo	*Escherichia coli*
134	Zoo	*Acinetobacter schindleri, Aeromonas bestiarum* (2), *Aeromonas trota* (2), *Enterobacter* (*Pantoea*) *agglomerans, Enterobacter gregoviae, Escherichia coli, Staphylococcus aureus, Staphylococcus chromogenes, Staphylococcus hominis*
188	Zoo	*Acinetobacter lwoffii* (3), *Aeromonas bestiarum*

Table 1: Bacteria identified in heart blood of dead big cats including leopards, lions and tigers.

Bacteria	Samples positive	Type of animals (case number)	Habitat (animals)
Escherichia coli	15	Leopard (71, 169), Lion (80, 121, 134), Tiger (15, 41, 55, 89, 90, 103, 105, 125, 202, 205)	Zoo (leopard, lions, tigers), Sanctuary (leopard, tigers)
Alcaligenes denitrificans	5	Lion (61), Tiger (54, 57, 103, 105)	Zoo
Enterobacter (*Pantoea*) *agglomerans*	5	Leopard (123), Lion (134), Tiger (11, 83, 115)	Zoo (leopard, lion, tiger), Sanctuary (tigers)
Enterococcus faecalis	5	Leopard (169), Tiger (103, 115, 169, 202)	Zoo (tigers), Sanctuary (leopard, tiger)
Proteus mirabilis	5	Leopard (71), Lion (98), Tiger (49, 55, 83, 103)	Zoo (leopard, tigers), Sanctuary (lion, tiger)
Aeromonas bestiarum	4	Leopard (123), Lion (134, 188), Tiger (15)	Zoo (leopard, lion), Sanctuary (tiger)
Enterococcus faecium	4	Leopard (169), Tiger (115, 169, 202)	Zoo (tigers), Sanctuary (leopard, tiger)
Staphylococcus aureus	4	Lion (134), Tiger (59, 105, 115)	Zoo
Aeromonas salmonicida ssp. *salmonicida*	3	Lion (114), Tiger (58, 83)	Zoo (Lion, tiger), Sanctuary (tiger)
Aeromonas trota	3	Leopard (123, 169), Lion (134)	Zoo (leopard, lion), Sanctuary (leopard)
Alcaligenes faecalis	3	Tiger (73, 115, 142)	Zoo
Streptococcus milleri	3	Tiger (49, 58, 103)	Zoo
Klebsiella pneumoniae ssp. *pneumoniae*	2	Leopard (169), Tiger (105)	Zoo (tiger), Sanctuary (leopard)
Proteus vulgaris	2	Tiger (89, 103)	Zoo, Sanctuary
Pseudomonas aeruginosa	2	Leopard (123), Tiger (117)	Zoo
Staphylococcus arlettae	2	Leopard (71), Lion (80)	Zoo
Staphylococcus chromogenes	2	Lion (134), Tiger (88)	Zoo
Staphylococcus intermedius	2	Leopard (169), Tiger (48)	Sanctuary
Staphylococcus lentus	2	Tiger (88, 115)	Zoo
Streptococcus pneumoniae	2	Lion (80), Tiger (80)	Zoo (lion), Sanctuary (tiger)
Streptococcus pyogenes	2	Lion (80), Tiger (59)	Zoo
Achromobacter xyloxidans	1	Lion (114)	Zoo
Acinetobacter calcoaceticus	1	Tiger (56)	Zoo
Acinetobacter lwoffii	1	Lion (188)	Zoo
Acinetobacter schindleri	1	Lion (134)	Zoo
Aeromonas hydrophila	1	Leopard (82)	Sanctuary
Aeromonas popoffii	1	Leopard (71)	Zoo
Bacillus cereus	1	Lion (114)	Sanctuary
Paenibacillus macerans	1	Tiger (100)	Zoo
Bacillus pantothenticus	1	Lion (114)	Zoo
Bacillus subtilis	1	Tiger (54)	Zoo
Citrobacter freundii	1	Tiger (15)	Sanctuary
Enterobacter gregoviae	1	Lion (134)	Zoo

Escherichia fergusonii	1	Tiger (202)	Zoo
Klebsiella oxytoca	1	Lion (80)	Zoo
Moraxella osloensis	1	Leopard (71)	Zoo
Moraxella phenylpyruvica	1	Leopard (71)	Zoo
Pragia fontium	1	Leopard (82)	Sanctuary
Raoultella terrigena	1	Lion (98)	Sanctuary
Staphylococcus capitis ssp. *capitis*	1	Lion (80)	Zoo
Staphylococcus epidermidis	1	Leopard (137)	Zoo
Staphylococcus haemolyticus	1	Tiger (103)	Zoo
Staphylococcus hominis	1	Lion (134)	Zoo
Streptococcus porcinus	1	Leopard (71)	Zoo

Table 2: Frequency of isolation of 44 species different bacteria from heart blood of dead big cats.

If we consider isolation of single type of bacteria as true case of septicemia then in 17 cases bacteria may be the cause of death. The most common isolation as single bacteria type was of *E. coli*, isolated from heart blood of a lion and four tigers, followed by isolation of *Alcaligenes denitrificans* (a lion, 1 tiger), *A. feacalis* (1 lion, 1 tiger) from two cases each while *Acinetobacter calcoaceticus, Bacillus cereus, Paenibacillus macerans, Enterobacter (Pantoea) agglomerans, Pseudomonas aeruginosa, Staphylococcus epidermidis, S. intermedius* and *Streptococcus pneumoniae* from one case each.

Of the 145 isolates of bacteria in the study multiple drug-resistance (MDR) was detected in 106 isolates (73.1%). The diversity of antibiotic drug-resistance can be imagined by the fact that 145 isolates belonged to 134 resistotypes (on the basis of resistance to antibiotics) and to any of the resistotypes no more than three isolates belonged. There was no significant difference in MDR occurrence among G+ve (73.91%) and G-ve (72.73%) bacteria isolated under the study. Similar was the observation for multiple herbal drug-resistances (Table 3).

Antimicrobials tested	Resistant G- strains	Resistant G+ strains	Comparison of G-ve versus G+ ve		
			Chi statistics	Odds ratio	Lower and upper limits at CI_{95}
Ajowan oil	17	17	0.009	0.35	0.16-0.78
Carvacrol	15	5	0.486	1.46	0.5-4.31
Cinnamledehyde	14	21	0.00004	0.20	0.09-0.44
Cinnamon oil	19	23	0.0001	0.24	0.11-0.51
Citral	41	24	0.225	0.65	0.32-1.31
Holy basil oil	38	28	0.011	0.40	0.2-0.82
Lemongrass oil	42	34	0.000	0.26	0.12-0.56
Thyme oil	17	9	0.727	0.85	0.35-2.09
Amoxycillin	67	29	0.583	1.23	0.59-2.55
Amoxycillin+ clavulanic acid	55	21	0.266	1.49	0.74-3
Ampicillin	63	10	0.000	6.30	2.8-14.18
Azithromycin	37	25	0.055	0.50	0.25-1.02
Cefepime	21	12	0.51	0.76	.34-1.72
Cefotaxime	25	9	0.45	1.39	0.59-3.28
Chloramphenicol	24	9	0.532	1.32	0.56-3.11
Ciprofloxacin	38	23	0.187	0.62	0.31-1.26

Cotrimoxazole	49	10	0.002	3.53	1.58-7.88
Erythromycin	92	26	0.000	10.11	3.85-26.52
Gentamicin	36	17	0.945	0.97	0.47-2.01
Imipenem	19	15	0.076	0.49	0.22-1.09
Meropenem	19	12	0.346	0.67	0.29-1.54
Nitrofurantoin	42	19	0.899	1.05	0.52-2.13
Penicillin	71	9	0.000	10.42	4.46-24.38
Piperacillin	52	18	0.13	1.72	0.84-3.51
Piperacillin+ tazobactam	19	1	0.01	10.69	1.38-82.51
Tetracycline	61	27	0.738	1.13	0.55-2.3
Tigecycline	24	9	0.53	1.32	0.56-3.11
Aztreonam	42	NT			
Colistin	31	NT			
Linezolid	NT	1			
Vancomycin	NT	5			
Multiple herbal antimicrobial resistant	39	25	0.091	0.55	0.27-1.11
Multiple antibiotic drug-resistant	72	34	0.881	0.94	0.43-2.08
Carbapenem resistance (CR)	19	21	0.001	0.28	0.13-0.61
Extended spectrum β-lactamase (ESBL) resistance	22	9	0.716	1.17	0.49-2.8

Table 3: Herbal and conventional antimicrobial resistance among 46 Gram-positive (G+) and 99 Gram-negative bacteria isolated from heart blood of dead leopards, lions and tigers (figures of Chi statistics in bold indicated significant difference among G+ and G- bacteria).

The bacteriological analysis of heart blood samples of leopards, lions and tigers revealed (Table 4) that there was no significant (p, >0.05) difference in occurrence of herbal antimicrobial resistance, antibiotic resistance, carbapenem resistant and ESBL producing strains among samples of different animals and of different locality. However, significantly high probability (p, ≤0.04) of the occurrence of strains resistant to multiple antibiotics (MDR), citral, tetracycline, nitrofurantoin, chloramphenicol and imipenem was recorded in samples from animals died in wildlife sanctuaries than those died in zoos. On the other hand, multiple herbal antimicrobial drug-resistance (MHDR) was significantly (p, ≤0.04) more common in bacterial strains isolated from leopard and tigers than those from heart blood of lions. The more MHDR in leopard and tiger origin strains was primarily due to the higher resistance in them to holy basil oil, cinnamaldehyde, lemongrass oil, citral and thyme oil.

Heart blood sources	Samples	Bacterial strains isolates	Average number of strains per sample	Average herbal antimicrobial drug resistance index (range)	Average antimicrobial drug resistance index (range)	Samples with strains having multiple herbalantimicrobial resistance	Samples with strains having multiple antimicrobial resistance	Samples with carbapenem drug resistant strains	Samples with ESBL producing strains
All wild big cats (Leopards 2, lions 2, tigers 6)	10	30 (G+11, G-19)	3	0.33 (0.0-0.75)	0.46 (0.14-0.81	5	10	3	5

All zoo big cats (Leopards 3, lions 7, tigers 20)	30	115 (G+35, G-80)	3.83	0.31 (0.0-0.88)	0.37 (0.0-0.86)	21	20	13	10
Zoo tigers	20	68 (G+22, G-46)	3.37	0.30 (0.0-0.88)	0.39 (0.0-0.86)	14	12	8	6
Wild tigers	6	16 (G+6, G-10)	2.67	0.25 (0.0-0.75)	0.37 (0.0-0.81)	2	4	2	4
All tigers (wild 6, zoo 20)	26	84 (G+28, G-56)	3.23	0.30 (0.0-0.88)	0.38 (0.0-0.86)	16	19	10	10
Lions (wild 2, zoo 7)	9	34 G+10, G-24)	3.78	0.28 (0.0-0.75)	0.39 (0.0-0.81)	5	6	3	2
Leopards (wild 2, zoo 3)	5	27 (G+8, G-19)	5.4	0.41 (0.0-0.88)	0.42 (0.10-0.81)	5	5	3	3

Table 4: Summary of the heart blood samples of leopards (5), lions (9) and tigers (26) died in wildlife sanctuaries (wild) and zoological parks (zoo) analyzed for presence of different types of bacteria (Gram positive, Gram negative) and bacterial susceptibility to herbal and conventional antimicrobials.

Of the 40 carbapenem resistant (CR) isolates identified from 16 (40.0%) heart blood samples (leopards 3, lions 4, and tigers10) in the investigation, 21 were negative for MBL. All MBL negative CR strains belonged to G+ve bacteria namely *Enterococcus faecalis* (8), *E. faecium* (4), *Staphylococcus aureus* (3), *S. chromogenes* (1), *S. epidemidis* (1), *S. intermedius* (1), *S. lentus* (2), and *Streptococcus pyogenes* (1) species. All the remaining 19 CR strains those produced MBL belonged to 8 species of G-ve bacteria viz., *Aeromonas bestiarum* (1), *A. trota* (2), *Alcaligenes faecalis* (3), *Enterobacter (Pantoea) agglomerans* (1), *Escherichia coli* (6), *Proteus mirabilis* (4), *P. vulgaris* (1), and *Raoultella terrigena* (1). Of the 19 strains those produced MBL, three also produced ESBL. In total 31 strains (21.37%) producing ESBL were detected in 15 (37.5%) samples in the study. The MIC of imipenem for MBL producer CR isolates ranged between 2 to 32 μg mL^{-1} while for those not produced MBL MIC ranged from 1.5 to 256 μg mL^{-1}, all the carbapenem susceptible isolates had imipenem MIC between 0.001 to 1.0 μg mL^{-1}.

Though there was no significant difference in probability of G+ve and G-ve strains being MDR, G+ve isolates had 3.54 times higher odds (CI 99%; 1.29-9.69) of being resistant to carbapenem drugs (meropenem and imipenem) than G-ve isolates (Table 3). Besides, G+ve strains had significantly (p, ≤0.01) higher odds of being resistant to ajowan oil, cinnamon oil, cinnamaldehyde, holy basil oil, and lemongrass oil, and higher odds of being susceptible to ampicillin, cotrimoxazole, erythromycin, penicillin G and piperacillin + tazobactam than G-ve bacteria isolated from heart blood of felids. For remaining herbal antimicrobials and antibiotics no such difference in susceptibility was evident among the two groups of bacteria. On G-ve isolates the most effective antibiotics were meropenem (80.81%), imipenem (80.81%), piperacillin+, tazobactam (80.81%), cefepime (78.79%), chloramphenicol (75.76%), tigecycline (75.76%) while on G-ve bacterial isolates the most effective antimicrobial were piperacillin+ tazobactam (97.83%), linezolid (97.83%), vancomycin (89.13%), penicillin (80.43%), chloramphenicol (80.43%), tigecycline (80.43%), cefotaxime (80.43%), ampicillin (78.26%) and cotrimoxazole (78.26%) inhibiting >75% of the isolates. Two of the herbal antimicrobials, thyme oil and carvacrol, were equally effective (inhibiting >80% isolates) against bacteria irrespective of their Gram staining character.

Discussion

In the present study, 16 (40.0%) of the 40 heart blood samples had one type of bacteria suggesting the probable role of the bacteria in septicemia. From majority of the samples (57.5%) multiple types of bacteria were isolated those may be either postmortem invaders, or result of contamination during sampling or may be the cause of septicemia by multiple bacteria at a time. In general, it is conceived that bacteremia/ septicemia often involves only a single type of organism, this concept prompts to conclude that a blood sample containing multiple organisms may be a contaminated one [17]. However, studies have shown that 6% to 21% cases of bacteremia may be polymicrobial, especially in immunocompromised, weak or high-risk patients [18]. Thus, detecting multiple types of bacteria in a blood sample is not always the result of sample contamination. However, to ensure true bacteremia and septicemia multiple blood samples collected from different site be tested and they must grow the same organism [10]. The collection of blood from multiple sites from a postmortem case is not practicable in most of the instances and the error can't be eliminated. Further, isolation of multiple bacteria from heart blood of 57.5% samples is a big figure indicating the possible contamination of heart blood samples due to one or more reasons. In clinical practice, single blood cultures are discouraged due to compromised specificity and sensitivity often difficult to interpret either the organism identified is a true cause of bacteremia or just a contaminant [10]. However, from postmortem cases collection of blood except from heart is often impossible and thus multiple blood samples may not be practical approach.

On applying the criteria that single type of bacteria isolated from blood may be the cause of septicemic death then in big cats the most common cause was of *E. coli* (5), followed by *Alcaligenes denitrificans* (2), *A. feacalis* (2), *Acinetobacter calcoaceticus* (1), *Bacillus cereus* (1), *Paenibacillus macerans* (1), *Enterobacter (Pantoea) agglomerans* (1), *Pseudomonas aeruginosa* (1), *Staphylococcus epidermidis* (1), *S. intermedius* (1), and *Streptococcus pneumoniae* (1). There may be several bacterial infections including tuberculosis, salmonellosis, bartonellosis, leptospirosis, psittacosis, glanders, pasteurellosis and septicemia due to *Staphylococcus aureus, Streptococcus pneumonia*e and *Escherichia coli* have commonly been reported to affect not only pet but wild felids [1-7,19-21]. Though other bacteria detected as possible cause fatal infections in humans including *A. faecalis* [22,23], *A. denitrificans* [24], *A. calcoaceticus* [25], *B. cereus* [26], *P. macerans* [27], *E. agglomerans* [28], *P. aeruginosa* [29], *S. epidermidis* [30], *S. intermedius* [31] and *S. pneumonia*e [32] but reported rarely from big cats.

Isolation of bacteria of 44 species from heart blood of big cats (18 species in leopards, 23 in lions and 25 in tigers) indicated diversity among bacteria which may be associated with septicemia/ bacteremia in big cats. The observations are in concurrence to earlier reports. A meta-analytical study on African lions and farmed lions' diseases indicated that a wide variety of microbes may be cause of illness, broadly, 63 pathogenic organisms recorded from lions belonged to 35 genera across 30 taxonomic families of which 56% were parasites, 27% were viruses and 17% were bacteria [7]. Big cats in captivity (zoos, lion farms etc.) often live under stressful environment and may become prone to acquire infection such as mange and ringworms besides life threatening tuberculosis and other contagious disease [33-36].

In India, mass mortality killing almost 50% of lion population in 2020 in Gir forest in past few years is of concern and diseases like rabies, pneumonia, urinary tract infection, anemia, hepatitis and multiple organ failure were held responsible for deaths. However, except rabies others are just the syndromes may be caused by a variety of pathogen. In an earlier mortality outbreak in 2018, 59 lions died out of 85 and canine distemper was diagnosed as the cause [37]. Canine distemper is reported to kill many lions and leopards in Serengeti in wild but secondary bacterial infection are often leads to lethality instead of the virus [5]. In leopards in Shivalik hills in India 8 of the 12 leopards died due to wound (caused by trapping injuries) complications [38]. Leopards also reported to die of electrocution on faulty high tension electricity lines passing through forests and in road accidents in India [39]. Though many leopards' deaths year after year in India are common [40], except pasteurellosis reported as cause of death in snow leopards [41,42], infections are not often considered as the cause of their death but injuries and fights among themselves or competing species animals are considered as the primary cause of their deaths. The present study indicated that in India too there may be number of bacteria invading the system of big cats may be leading to their mortality but needs a systemic examination [38].

Frequent occurrence of multiple drug resistant bacteria (>73%) in heart blood of big cats observed in the present study is alarming.

Similar high prevalence MDR in bacteria has been reported frequently from wild life, rescued animals and zoo animals in India [43-45] and abroad [46-50]. Though carbapenem resistance is reported in bacteria isolated from wild life, zoo animals, rescued animals [43,45,51] and domestic animals [52] in India, isolation of carbapenem drug-resistant bacteria from 16 (40.0%) of the 40 samples tested is of serious public health concern as carbapenems are considered as the last line drugs available for therapeutic use in humans and are not recommended or rarely used in animals. In G+ve bacteria resistance to imipenem and meropenem but sensitivity to penicillin G observed in the present study might be due to mutation in different penicillin binding protein (PBPs) targets used by the two groups of antibiotics. The similar pattern i.e., resistance to carbapenem but susceptible to penicillin G has been observed among a few G+ve bacteria especially in *Streptococcus pneumoniae* [53,54] but rare in other bacteria observed to posses the characteristic in the present study and penicillin binding protein profile of such isolates may elucidate the reason behind the observation.

In the study none of the herbal antimicrobial was inhibitory to all of the 145 bacteria isolated in the study and 64 (44.14%) had multiple herbal antimicrobial resistance. Herbal antimicrobials are often considered as an alternative to antibiotics but resistance to herbal antimicrobials is also not uncommon [55,56], and is reported to be an emerging problem [57].

Conclusion

The study concluded multiplicity of bacteria in heart blood of dead leopards, lions and tigers in zoo and in wildlife sanctuaries indicating that except E. coli nothing is common or more important as cause of bacteremia or septicemia. Isolation of multiple bacterial types from 57.5% samples suggests need for aseptic and timely collection of blood samples to understand the true etiology of fatality among big cats. Detection of MDR, ESBL and CR bacteria from 25%, 37.5% and 40% samples is alarming because of chances of spreading of AMR in the environment from animals suffering from infections with MDR strains and died in wild.

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Conflict of Interest

None to declare.

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