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Research Article

Antimicrobial Susceptibility Patterns of Bacteria Isolated from Aborted Foetuses of Lions (*Panthera leo*) and Tigers (*Panthera tigris tigris*)

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Abstract

Abortions are multietiological disorders of pregnancy interfering with reproduction, and bacteria are often the most common cause of in-utero death of foetii. The study was conducted to understand the bacteria associated with abortion and foetal death in big cats. Bacteriological analysis of aborted foetii samples (heart blood, stomach contents, liver, spleen, kidneys and lunges etc.) from lions (two) and tigers (four) revealed presence of bacteria of 11 different species viz., *Aerococcus* spp., 10; *Alcaligenes faecalis,* 1; *Bordetella bronchiseptica,* 2; *Enterobacter agglomerans,* 3; *Enterococcus faecalis,* 1; *Enterococcus malodoratus,* 4; *Escherichia coli,* 11; *Falvobacterium aquatile,* 2; *Klebsiella pneumoniae* ssp. *pneumoniae,* 4; *Pasteurella canis,* 4 and *Streptococcus milleri,* 1 from aborted foetii of big cats. Antimicrobial susceptibility patterns of the bacteria isolated from contents of aborted foetii indicated that 65.12% bacterial isolates were multiple drug-resistant (MDR), 60.47% had multiple herbal antimicrobial drud-resistance (MHDR), 13 isolates were resistant to carbapenems and 12 produced extended spectrum β-lactamases. All Gram-negative bacterial isolates were susceptible to colistin and all Gram-positive isolates to linezolid but none of the other antibiotic could inhibit ≥80% of the isolates. However, all the isolates were susceptible to thyme oil and carvacrol and only 2.35%, 4.65%, 9.30%, 16.28% and 30.23% were resistant to cinnamaldehyde, cinnamon oil, holy basil oil, ajowan oil and lemongrass oil, respectively. In four cases of abortion in tigresses, *Escherichia coli, Bordetella bronchiseptica, Pasteurella canis* and *Flavobacterium aquatile* might be associated with abortions. In lionesses, *E. coli* appeared to be important bacteria associated with abortion. The study concluded that many different types of MDR bacteria may be invading the foetii. The study clearly indicated that just isolation and identification of bacteria from aborted foetii may not be conclusive in determining the cause of abortion in lions and tigers and microbiological results must be interpreted carefully.

Keywords: Big Cats; *Escherichia coli*; *Bordetella bronchiseptica*; *Pasteurella canis*; *Flavobacterium aquatile; Klebsiella pneumoniae*; *Streptococcus milleri*; *Enterococcus faecalis*; MDR; Carbapenem-resistance

Introduction

Delivery of dead babies (usually intra-uterine) is defined as abortion when the babies are aborted nearing full term often designated as stillbirths. There may be innumerable reasons for abortions but septic abortions resulting from bacteraemia are of major concern. Common causes of bacteraemia leading to abortions include *Brucella* spp., *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris*, hemolytic streptococci,

staphylococci, *Arcanobacterium pyogenes*, *Bacillus* spp., *Pasteurella* spp., *Pseudomonas* spp., *Salmonella* spp [1-3], and sometimes *Clostridium perfringens, Fusobacterium* spp., *Flavobacterium* spp., *Bordetella* spp. too [4,5]. Lot many studies and investigation reports on abortions are from pets and domestic animals [1-5], and causes of abortion in zoo animals are rarely reported. The present report describes the four abortion cases in zoo tigresses over the last three years. Though it is difficult to monitor the lions and tigers for preabortion infection and thus the timely administration of antibiotics to treat the infections, emergence of antimicrobial drug resistance (AMR) is a global health threat and reported commonly in bacteria affecting wild animals too [6] and further complicate the problem. The present study was conducted on samples of aborted foetii from lions and tigers to ascertain the proble bacterial causes of abortion in big cats and determining their antimicrobial susceptibility to understand the AMR patterns of bacteria associated with aborted foetii.

Materials and Methods

Zoo veterinarians submitted samples from aborted foetuses including heart blood, tracheal swabs, tissues from lung, spleen, liver, kidneys and stomach contents. The samples were transported on ice and were processed in the Clinical Epidemiology Laboratory of ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, India soon after receipt of the samples for bacteriological analysis as per the standard procedure for detection of anaerobic, microaerobic and aerobically growing bacteria. For isolation of bacteria from samples, 1 mL of blood/stomach contents were transferred to a 100 ml thioglycollate medium (BBL-Difco, USA) and incubated at 37°C for 24 h. For tissue samples, approximately 100 mg of tissue aseptically taken was homogenized in 1 ml buffered peptone water (BPW, BBL-Difco, USA), transferred to 10 mL of thioglycollate medium and incubated for 24 h at 37°C. The growth from the thioglycollate medium was streaked onto 5% sheep defibrinated sheep blood agar (BBL, Diffco, USA) plates in triplicate. One set was incubated at 37°C for 24 h aerobically, another microaerobically and the third one anaerobically. The tracheal swab samples were transferred directly to thioglycolate medium and processed as other samples. All plates were observed for isolated colonies. All different types of colonies (3-5) were picked up and re-streaked onto nutrient agar plates for purification and incubated at 37°C for 24 h. The pure cultures were tested for morphological, culture, staining and biochemical characteristics using standard protocols [7,8]. Thereafter, bacterial isolates were classified up to genus and species levelsusing criteria laid in Bergey's Manual of Determinative Bacteriology [9]. All isolates were also confirmed for their identity using MALDI-ToF MS performed with a MALDI Biotyper Sirius system (Bruker Daltonics).

All isolates were tested for their antimicrobial susceptibility using disc diffusion assay on Mueller Hinton agar as per CLSI guidelines [10,11] using antibiotic discs procured from Difco (USA) for ampicillin (10 µg), amoxycillin+ clavulanic acid (50+10 µg), amoxycillin (30 µg), amoxycillin+ sulbactam (30+15 µg), aztreonam (30 µg), cefepime (30 µg), cefotaxime (30 µg), cefotaxime+ clavulanic acid (30+10 µg), cefoxitin (10 µg), chloramphenicol (25 µg), ciprofloxacin (10 µg), colistin (10 µg), cotrimoxazole (25 µg), erythromycin 15 µg), fosfomycin (50 µg), gentamicin (30 µg), linezolid (30 µg), meropenem (10 µg), nitrofurantoin (300 µg), tetracycline (30 µg), and vancomycin (30 µg). Resistance patterns of different isolates (resistograms) were deduced in accordance of for CLSI (CLSI, 2015a,b) after measuring diameter of the growth inhibition zone in mm. Antimicrobial susceptibility to colistin and aztreonam were determined for Gram-negative and linezolid and vancomycin for Gram-positive bacterial isolates only. The potential to produce extended-spectrum β-lactamase (ESBL) and Metallo-β-lactamase (MBL) was determined by a double-disc diffusion assay [10,11]. Susceptibility for herbal antimicrobials including ajowan (*Tachyspermumammi*) seed oil, betel (*Piper betel*) leaf oil; carvacrol, cinnamaldehyde, cinnamon (*Cinnamomum zeylanicum*) bark oil, citral, guggul (*Commiphora wightii mukul*) oil; holy basil (*Ocimum sanctum*) oil, lemongrass (*Cymbopogon citrates*) oil, sandalwood (*Santlum album*) oil, and thyme (*Thymus vulgaris*) oil (procured from Sigma (USA) and Naga Fragrence Ltd. Dimapur) was determined by disc diffusion assay using discs containing 1µL of the test herbal compound [12]. For all isolates, resistograms were prepared and compared to determine the similarity among different isolates.

Results and Discussion

Abortion cases in tigresses

Case number 1 (150). *Escherichia coli* with the same resistogram (Table 1) were isolated from the heart blood, liver and spleen of the aborted foetus indicating that the *E. coli* might be the probable cause of abortion. *Escherichia coli* have often been reported as

the cause of abortion in different types of animals and pets [1-5]. Besides, *Enterococcus malodoratus* was also isolated from the liver of the aborted foetus but its absence from other organs and heart blood indicated that it might be either an environmental contaminant or a secondary invader. As an opportunistic pathogen, *E. malodoratus* has been reported to cause several ailments but rarely as a primary causal agent [13].

Sample Number	Source of isolation from aborted foetuses (sample)	Bacteria identified	Herbal drug resistance pattern	Antibiotic resistance pattern
Samples of aborted foetii of tigresses				
150	Heart blood	*Escherichia coli*	Ctr, GO, SWO	Nil
150	Liver	*E. coli*	Ctr, GO, SWO	Nil
150	Liver	*Enterococcus malodoratus*	BLO, GO, SWO	Co, G
150	Spleen	*E. coli*	Ctr, GO, SWO	Nil
151	Heart blood	*Bordetella bronchiseptica*	Ctr, GO, SWO	A, AMX, AMC, AMS, AT, CPM, CTX, CTX-CLA, CFX, CTR, MP, T
151	Spleen	*B. bronchiseptica*	Ctr, GO, SWO	A, AMX, AMC, AMS, AT, CPM, CTX, CTX-CLA, CFX, CTR, MP, T
151	Spleen	*E. malodoratus*	BLO	Nil
151	Liver	*E. malodoratus*	BLO, GO, SWO	Co, G
151	Liver	*Enterobacter agglomerans*	Ctr, LGO, GO	Co, E, T
151	Lung	*E. malodoratus*	Ctr, BLO, LGO	Nil
151	Lung	*E. agglomerans*	Ctr, LGO, GO	AT, E
167	Liver	*Pasteurella canis*	Nil	T
167	Liver	*Aerococcus* spp.	Citral, LGO, MEO	A, AMX, AMC, Cf, CAZ, CAC, CTX, CTX-CLA, CTR, CFX, CPM, MP, T, Va
167	Stomach contents	*P. canis*	Nil	T
167	Stomach contents	*Aerococcus* spp.	Ctr, BLO, HBO, LGO, GO	A, Amx, AMC, CAZ, CAC, CTX, CTX--CLA, CTR, T, Va
167	Heart blood	*Aerococcus* spp.	BLO, GO	A, CAZ, CAC, CTX, CTX-CLA, CTR
167	Heart blood	*Aerococcus* spp.	Nil	A, AMX, AMC, CAZ, CAC, CTX, CTX-CLA, CTR, CPM, T, Va
167	Heart blood	*P. canis*	Nil	T
167	Heart blood	*Alcaligenes faecalis*	SWO	AT, Co, CTX, CTX-CLA, CTR, CFX, FO, T
167	Lung	*P. canis*	Nil	T
167	Lung	*Aerococcus* spp.	Ctr, BLO, GO, MEO	CFX, E, Va
167	Spleen	*P. canis*	Nil	T
167	Spleen	*Aerococcus* spp.	GO, MEO	Va
168	Spleen	*Flavobacterium aquatile*	Nil	A, AT, C, T
168	Heart blood	*F. aquatile*	Nil	A, AT, C, T
Samples of aborted foetii of lionesses				
170	Spleen	*E. coli*	BLO, GO, LGO, SWO,	A, AMX, AMC, AMS, AT, Co, Cf, CTX, CTX-CLA, CFX, CPM, E, FO, G, Nf, T

170	Trachea	*E. coli*	BLO, GO, LGO, SWO,	AMX, AMC, AMS, AT, Co, Cf, CTX, CTX-CLA, CFX, CPM, E, FO, G, MP, Nf, T
170	Trachea	*E. coli*	AO, GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CTX, CTX-CLA, CFX, CPM, E, FO, G, MP, Nf, T
170	Trachea	*E. coli*	AO, GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CTX, CTX-CLA, CFX, CPM, E, FO, G, Nf, T
170	Trachea	*E. coli*	AO, GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CTX, CTX-CLA, CFX, CPM, E, FO, G, Nf, T
170	Liver	*Klebsiella pneumoniae ssp. pneumoniae*	Ctr, GO, LGO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CFX, E, FO, G, Nf, T
170	Spleen	*K. pneumoniae* ssp. *pneumoniae*	GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CFX, E, FO, G, Nf, T
170	Lumg	*K. pneumoniae* ssp. *pneumoniae*	AO, GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CFX, CTX, CTX-CLA, CPM, E, FO, G, Nf, T
170	Heart blood	*K. pneumoniae* ssp. *pneumoniae*	AO, GO, SWO	A, AMX, AMC, AMS, AT, Co, Cf, CFX, CPM, CTX, CTX-CLA, E, FO, G, MP, Nf, T
170	Trachea	*Streptococcus milleri*	AO, GO, SWO	A, AMX, AMC, AMS, Co, Cf, CFX, CPM, E, FO, G, Nf, T
207	Spleen	*E. coli*	Ctr, GO, HBO, LGO, SWO	A, AMX, AMC, AMS, Co, CFX, CTX, CTX-CLA, E, FO, Nf
207	Liver	*E. coli*	Ctr, GO, HBO, SWO	AMX, AMC, AMS, CFX, CTX, CTX-CLA, E, FO, Nf
207	Lumg	*E. coli*	Ctr, GO, LGO, SWO	A, AMX, AMC, AMS, Co, Cf, CTX, CTX--CLA, E, FO, Nf
207	Lumg	*E. agglomerans*	Ctr, GO, LGO, SWO	A, AMX, AMC, AMS, AT, CPM, CTX, CTX-CLA, E, FO, Nf, T
207	Liver	*Enterococcus faecalis*	AO, CO, Ctr, CNH, GO, HBO, LGO, SWO	Nil

Table 1: Bacteria isolated from different samples of aborted foetuses of four tigresses (*Panthera tigris tigris*) and two lionesses (*Panthera leo*) their antimicrobial resistance patterns.

Herbal antimicrobial discs used: AO, ajowan oil (1 mg); BLO, betel leaf oil (1 mg); C, carvacrol (1 mg); CNH, cinnamaldehyde (1 mg); CO, cinnamon oil (1 mg); Citral (1 mg); GO, guggul oil (1 mg); HBO, holy basil oil (1 mg); LGO, lemongrass oil (1 mg); SWO, sandalwood oil (1 mg) and TO, thyme oil (1 mg).

Antibiotic discs used: A, ampicillin (10 µg); AMC, amoxicillin+ clavulanic acid (50+10 µg); AMX, amoxicillin (30 µg); AMS, amoxicillin+ sulbactam (30+15 µg); AT, aztreonam (30 µg); CPM, cefepime (30 µg); CTX, cefotaxime (30 µg); CTX-CLA, cefotaxime+ clavulanic acid (30+10 µg); CFX, cefoxitin (10 µg); CAZ, ceftazidime (30 µg); CAC, ceftazidime+ clavulanic acid (30+10 µg); CTR, ceftriaxone (10 µg); C, chloramphenicol (25 µg); Cif, ciprofloxacin (10 µg); Cl, colistin (10 µg); Co, cotrimoxazole (25 µg); E, erythromycin 15 µg); FO, fosfomycin (50 µg); G, gentamicin (30 µg); Lz, linezolid (30 µg); MP, meropenem (10 µg); NF, nitrofurantoin (300 µg); T, tetracycline (30 µg); Va, vancomycin (30 µg)

Note: Colistin and aztreonam were used for susceptibility testing of Gram-negative and linezolid and vancomycin for Gram-positive bacterial isolates only.

Case number 2 (151). *Bordella bronchiseptica* isolates having the same resistogram (Table 1) were isolated from the heart blood and spleen but not from liver and lung tissues of the aborted foetus while *E. malodoratus* isolates with altogether different resistogram were detected from spleen, lung and liver of the aborted foetus and *Enterobacter agglomerans* with dissimilar resistogram from liver and lung tissues of the same foetus. Due to the similarity in resistogram of all *B. bronchiseptica* isolates it might be considered the main causal agent of abortion. However, *E. agglomerans* have also been reported to be a cause of abortion in animals [14,15].

Both cases (case 1 and case 2) were from the same zoo and with a difference of only one day. In both cases *E. malodoratus* with a similar resistogram that too from the same organ i.e., liver of aborted foetuses indicated some association with the disease. However, in the 2nd case, *E. malodoratus* with no antibiotic resistance were isolated from the spleen and lung also and were different from those isolated from livers of the two aborted foetuses. Earlier studies have reported frequent antibiotic resistance among enterococci including *E. malodoratus* [16] the infrequent use of antibiotics in zoo tigers may be responsible for antimicrobial drug-susceptible isolates. However, *B. bronchiseptica* with a wide-spectrum antimicrobial resistance, possessing MBL potential, is of a public and veterinary health concern as most of *B. bronchiseptica* are reported to be susceptible to carbapenems and such a broad spectrum resistance is of rare occurrence in *Bordetella* [17]. Though production of ESBL has recently been reported by *B. bronchiseptica* of animal origin leading to resistance towards some β-lactam antibiotics including penicillins and cephalosporines as observed in the present study [17]. Though *B. bronchiseptica* is known to cause a variety of respiratory tract infections and soft tissue infections in animals and pets, have rarely been reported as a cause of abortion [17] except in guinea pigs [18]. It appears to be the first report of *B. bronchiseptica* induced abortion in tigress.

Case number 3 (167). From the aborted foetus' stomach contents, heart blood, lung, and spleen *Pasteurella canis* with the same antibiograms (Table 1) susceptible to most of the antimicrobials but resistant to streptomycin and tetracycline indicated that it might be the causal agent of abortion. However, *Aerococcus* strains of different resistance patterns were detected from the liver, stomach contents, heart blood, lung and spleens probably may not be the cause of disease but might be a contaminant from the environment or commensals invading samples during collection. Though aerococci have shown an emerging pathogen potential to cause a variety of illnesses in humans and animals have rarely been associated with abortion [19,20]. On the other hand, *Pasteurella* strains of different species are often reported in association with abortion in animals [21-23]. Besides aerococci and *P. canis*, multiple drug-resistant *Alcaligenes faecalis* were also detected in heart blood of the aborted foetus. Though *Alcaligenes* strains are often reported to carry multiple drug resistance traits [24], have rarely been reported in the association with abortion [25].

Case number 4 (168). From aborted foetus' spleen and heart blood only one type of bacteria, *Flavobacterium aquatile,* was detected (Table 1) in pure culture indicating the role of the bacterium in abortion in tigress in late pregnancy. All the isolates had similar resistogram means belonging to the same strain. Multiple drug resistance to commonly used antibiotics in all the isolates of *Flavobacterium aquatile* may be of public health concern. Most of the flavobacteria are commensal in marine life. *Flavobacterium aquatile* was first isolated from freshwater from a well in Kent, England and had rarely been reported to cause abortion. However, several *Flavobacterium* strains have been reported to be present in 6.89% of the placenta of aborted cattle [5]. The association of *F. aquatile* with abortion in tigress appears to be the first case.

Cases of abortion in lionesses

Case number 1 (170). *Escherichia coli* with MDR and resistant to carbapenems producing MBL and with the same antibiograms were detected in tracheal swab and spleen of the aborted foetus but from tracheal swab *E. coli* with three different antibiograms were also detected (Table 1). Tracheal swab also had a MDR *Streptococcus milleri* (Figure 1). Besides, MDR *Klebsiella pneumoniae* ssp. *pneumoniae* were detected in liver (ESBL positive), spleen (ESBL positive), lung and heart blood (MBL positive) of the same foetus but all of different antimicrobial susceptibility patterns mean all different strains. Both, *E. coli* and *K. pneumonia*e, are reported to cause abortions in felids and other animals [1-5] but with the present investigation it is not possible to reach at definitive diagnosis.

Case number 2 (207). The liver, spleen and lung sample of the aborted foetus had three different strains of MDR *E. coli* all with

Figure 1: *Streptococcus milleri* isolated from tracheal swab of aborted lion foetus, Loeffler's methylene blue stains 1000x.

different antiogram (Table 1) but none produced ESBL or MBL. Besides, *Enterococcus faecalis* susceptible to all tested antibiotics was detected in liver of the aborted foetus and a MDR strain of *E. agglomerans* fron lung. Though *E. coli* is a well known cause of abortion [1-5], *E. agglomerans* are rarely reported cause of abortion [14,15].

Antimicrobial resistance among bacteria isolated from aborted foetii of lion and tigers. The antimicrobial susceptibility patterns of the bacteria isolated from aborted foetii (Table 1) indicated that bacteria of the same species with different antibiograms were present in same organ or different organs of aborted foetii and bacteria of different species from the same or different organs may also be present. Of 43 isolates of bacteria from aborted foetii sample 65.12% were multiple drug-resistant (MDR), 60.47% had multiple herbal antimicrobial drud-resistance (MHDR), 13 isolates were resistant to carbapenems and 12 produced extended spectrum β-lactamases. Of the 13 carbapenem resistant bacteria 7 (*E. coli* 4, *B. bronciseptica* 2, *K. pneomoniae* 1) produced MBL while six (*Aerococcus* spp. 5, and *S. milleri* 1) were negative for MBL. Production of MBL by Gram negative bacteria including those from wild life has been reported earlier too [6]. Resistance to carbapenems without production of MBLs is common among Gram-positive bacteria especially in streptococci, and enterococci [26,27] but rarely reported in *Aerococcus* species. All Gram-negative bacterial isolates were susceptible to colistin and all Gram-positive isolates to linezolid but none of the other antibiotic could inhibit ≥80% of the isolates. However, all the isolates were susceptible to thyme oil and carvacrol and only 2.35%, 4.65%, 9.30%, 16.28% and 30.23% were resistant to cinnamaldehyde, cinnamon oil, holy basil oil, ajowan oil and lemongrass oil, respectively. Though herbal antimicrobials are considered as an alternative for antibiotics especially when AMR is emerging fast [28], resistance to some of the herbal antimicrobials is not uncommon [6,12] and is said be emerging among bacteria causing infections in animals [29].

The study revealed carriage of several zoonotically important MDR bacteria on aborted foetii of big cats which may risk not only wildlife and zoo animals' but animal caretakers too. Most of the bacteria detected on aborted foetii might be associated with serious ailments not only in animals but also in humans like osteomyelitis [30], diarrhoea and other enteric infections [31,32], and septicemia and sepsis [33,34]. Thus hygienic handling of abortions in big cats is as important as handling of abortion cases in domestic animals [35].

Conclusion

The study concludes that from aborted foetii of tigers and lions several different types of bacteria may be isolated either in pure culture from different organs or multiple types of bacteria from the same organ thus it is not possible to assign a bacteria as stand alone cause of abortion or associated with abortion unless detailed studies supported with serology and histopathology are done in association with a clinical observation. The study suggests systematic studies on causes of abortions in tigers and lions to conserve the most beautiful large felids.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data that support this study will be shared upon reasonable request to the corresponding author.

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