Aerobic Microbiome of Vagina of Apparently Healthy Pregnant Large Black Sows in Nagaland and Antimicrobial Resistance in Isolates

Bhoj R. Singh[*,1] and Ngullie Ebibeni[2]

[1]*ICAR Research Complex for NEH Region, Jharnapani, Nagaland 797 106, India*

[2]*Krishi Vgyan Kendra, Dimpur, Jharnapani, Nagaland 797 106, India*

Abstract: For understanding the culturome of vagina of pre-parturient sows, 74 swab samples were analysed for aerobic mesophilic bacteria through pre-enrichment in buffered peptone water at 37°C for 6 h then streaking on blood agar, trypticase soy agar (TSA) and Hektoen enteric agar (Hi-Media) plates and incubation at 37ºC for 24-48 h. Gram negative bacteria (GNB) were detected in 56 samples while Gram positive bacteria (GPB) in 28 samples. Of the 115 isolates identified belonging to 30 species of 16 different genera, 33 were GPB and 82 were GNB. *Aeromonas* (39.2%) were the most common isolates followed by *Enterococcus* (37.8%), *Klebsiella* (18.9%), *Escherichia* (10.8%), *Citrobacter* (8.1%), *Pragia* (6.8%) and *Proteus* (6.8%). Only eight were sensitive to all 8 antimicrobials tested using disc diffusion assay on Mueller Hinton agar in the study while rest 107 could be classified into 25 antibiogram types (ATs). All GPB and 51.2% of GNB isolates were resistant to nalidixic acid, 75.6% to ampicillin. Ciprofloxacin inhibiting 99.2% isolates was most effective antimicrobial followed by gentamicin (96.5%) and ceftriaxone (95.7%). Resistance against herbal antimicrobials was detected in 87.8%, 87%, 78.3%, 76.5% and 35.7% of isolates against *Artemisis vulgaris* essential oil, sandal wood oil, eucalyptus gum, patchouli essential oil and lemon grass oil (LGO), respectively. Except for LGO, resistance to herbal antimicrobials was more common in isolates of aerobic bacteria of sow vagina than the least effective antimicrobial drugs nalidixic acid (65.2%) and ampicillin (60.9%). Multiple herbal antimicrobial resistance (MHAR) was detected in 89.6% strains tested, while multiple antibiotic resistance in 28.7% strains. Enterococci had higher probability (P, 0.02) of MHAR among GPBs while among GNBs members of Enterobacteriaceae (92 %) were more frequent carriers (P, 0.07) of MHAR than aeromonads (78.1%). The study indicated that aerobically culturable microbiome which is constituted exclusively by facultative anaerobe might be important due to their ability as opportunistic pathogens and ability to grow and compete with anaerobic flora of healthy vagina. The study revealed that in pre-parturient sow vagina the aerobically culturable microbiome is diverse and many potentially pathogenic bacteria present may harbour multiple drug resistant bacteria necessitating the proper inference from microbial analysis tests.

Keywords: Vaginal-microbiome, *Edwardsiella tarda, Aeromonas, Klebsiella pneumoniae*, antimicrobial-drug resistance, MDR, herbal antimicrobial resistance, MHAR.

1. INTRODUCTION

Vaginal bacterial flora in mammals play an important role in maintenance of functionality of reproductive tract [1], local mucosal immunity [2] and transfer of microbiota to progeny (during birth) required for peri-natal protection from infections [3-6]. Vaginal bacterial flora is dynamic, complex and ill understood [7, 8], but vital. Normal functionality of vagina and reproduction failure has often been associated with change in normal microbiota in the vagina [7, 9, 10]. The role of commensal bacteria in vagina in the maintenance of a healthy reproductive tract and also in precipitation of some vaginal pathology is quite understood [7-9, 11]. Recent observations have shown that transfer of bacteria to progeny during natural birth takes place in birth canal (vagina) and ensure good health to neonates [3-5, 12]. Studies have been conducted on commensal / normal microbiological flora of reproductive tract in women [1], mares [9, 10], bitches [11, 13], sows [14], swamp buffaloes [15], mithuns (*Bos frontalis*) [16], guinea pigs [17], cows [18] and even in lizards [6] at different stages of reproduction.

The lower reproductive tract consisting vagina and cervix are the important source of microbiome acquired by piglets and other newborns during their travel through birth canal [19]. The acquision of healthy microbiome by infant leads to good health, and healthy microbiome in reproductive tract is the key of lifelong reproductive efficiency [20]. However, bacterial flora of vagina is always in dynamic state due to its opening to the environment (affected by animal husbandry practices, climate and geography), hormonal variation of reproductive cycle and vicinity to urethra and anus; the two most potent sources of bacteria in reproductive tract [21-23]. Besides, antimicrobial therapy is another important determinant of microbial flora in vagina and other body parts leading to major microbial shift [21, 24]. Therefore, study of vaginal microflora at different farms is quiet important to understand economic animal husbandry. Understanding of commensal bacteria in vagina of sows in late pregnancy may be important to

*Address correspondence to this author at the Head Division of Epidemiology, Indian veterinary Research Institute, Izatnagar-243 122, India;
Tel: +91-8449033222; E-mail: brs1762@gmail.com

E-ISSN: 2310-8703/16

understand the reproductive efficiency and for improving peri-natal management of piggeries. However, there appears to be scanty information about commensally occurring bacteria in vagina of healthy animals in late pregnancy. Large black pig is well adapted and frequently preferred breed of pigs reared in Nagaland [25]. To meet the need of piglets for farmers rearing pigs, a Large Black (LB) breeding seed farm is established in Jharnapani near Dimapur in Nagaland. Occurrence of aerobically growing bacteria in vagina of apparently healthy LB sows was studied in their late pregnancy to reveal aerobic microbiota of apparently healthy pig vagina. Although anaerobes are quite important as inhabitants of deep vagina, and they outnumber aerobes, the importance of facultative anaerobes growing aerobically cannot be ignored due to their opportunistic pathogenic potential and greater ability to modulate healthy vaginal flora [7, 8]. The facultative anaerobes are important due to their ability to compete with strict anaerobic bacteria (often considered normal inhabitants) in vagina of pregnant animals. In animals and specifically in sows anaerobic bacteria have less commonly been reported to cause infection in post-natal life in infants and also in sows [14, 22-24], therefore, the study is targeted to study aerobically growing bacteria in sow vagina.

2. MATERIALS AND METHODS

2.1. Vaginal Swabs

Vaginal swab (VS) samples were collected from 74 apparently healthy Large Black (LB) pregnant sows (90-100 days of gestation) maintained for breeding at Pig Seed Breeding Farm at Jharnapani in Nagaland state. The study was undertaken from over a period of 3 months (March to May). Animals were adequately restrained and peri-vaginal area was scrubbed to clean with warm water and then with a strong non-irritant antiseptic (Triclogel, HiMedia, Mumbai). Sterile swab sticks (Hi-Media) were inserted deep (10-12 cm) into the vagina with the vaginal labia gently parted apart and avoiding contact with clitoral region and urethral orifice. All possible steps were taken to avoid any environmental and peri-vaginal contact of swabs to prohibit detection of contaminants. The swabs were transferred to sterile screw capped test tubes and labelled. The samples were brought to the laboratory within one hour of collection.

2.2. Isolation and Identification of Bacteria

Each swab was inoculated into 10 ml buffered peptone water (BBL BD, USA) as soon as it reached the laboratory and incubated at 37^{o}C for 6 h. Then a loopful broth from the tube was inoculated onto 5% sheep blood agar (BA), trypticase soy agar (TSA) and Hektoen enteric agar (Hi-Media) plates and then incubated at 37ºC for 24-48 h. From plates positive for growth, 3-10 well isolated colonies were picked up and re-streaked onto BA plates for purification. Pure cultures were identified on the basis of Gram staining, morphological, growth and biochemical characteristics using standard procedures [26, 27].

2.3. Antimicrobial Drug Sensitivity Assay

All the isolates were tested for their sensitivity using disc diffusion methods as per CLSI [28] guidelines on Mueller Hinton agar (Hi-Media), using ampicillin 25μg (A), ceftazidime 30μg (Ca), chloramphenicol 30μg (C), ciprofloxacin 10μg (Cf), cotrimoxazole 25μg (Co), gentamicin 30μg (G), nalidixic acid 30μg (Na) and tetracycline 30μg (T) discs (Hi-media). Diameter of zone of growth inhibition around disc was measured in mm and isolates were classified as sensitive or resistant according to CLSI [28] standards. Multiple drug resistance (MDR) was noted if the test strain was resistant to three or more of the eight antimicrobials tested. A reference *E. coli* K12 strain (E-382), sensitive to all antibiotic was used as control. Besides, all the gram positive isolates were also tested for their sensitivity to clindamycin 2μg, erythromycin 15μg, oxacillin 1μg and vancomycin 30μg using disk diffusion assay [28].

2.4. Herbal Antimicrobial Sensitivity Assay

All the isolates were also tested for sensitivity for herbal antimicrobials using disc diffusion methods [29]. The herbal antimicrobial discs (containing 1 mg of herbal antimicrobial per disc) of sandalwood oil (SWO), patchouli essential oil (PEO), *Artemesia vulgaris* essential oil (AVEO), lemongrass oil (LGO), and eucalyptus gum (EG), all procured from Shubh Flavours and Fragrance P. Ltd (New Delhi) were used in the study. Bacteria were classified sensitive if any measurable zone of inhibition around discs of herbal ingredient was observed.

2.5. Statistical Analysis

For finding difference in prevalence and or co-occurrence of different bacteria in vaginal swabs, and for comparing the results of sensitivity patterns of bacteria isolated from VS of pregnant sows Chi-square (χ^2) test was used.

3. RESULTS

3.1. Bacteria Isolated from Vaginal Swabs

Of the 74 VS samples, one or more type of bacteria could be detected from 56 (75.7%) samples. From 18 (24.3%) samples no aerobically growing mesophilic bacteria could be detected (Table **1**). All the bacteria isolated from pig vagina in the study were also able to grow anaerobically and belonged to facultative anaerobic genera. In most of the VS samples either only one (27%) or two (35.1%) types of bacteria were detected but in some of the samples (13.6%) more than two and up to eight types of bacteria could be detected (Table **1**).

Of the 115 isolates identified from 56 samples, 33 were Gram positive (GPB) and 82 were Gram negative bacteria (GNB). On further characterization, bacteria isolated from vagina of sows were classified into 30 species of 16 different genera (Table **2**). The GPBs were detected in only 28 samples while GNBs could be isolated from all 56 samples positive for bacteria. The GPBs were always detected along with GNBs in the samples positive for bacteria. On the other hand in 28 (37.8%) samples only GNBs were detected. Probability of detection of aeromonads (39.2%) in VS was the maximum followed by *Enterococcus* (37.8%), *Klebsiella* (18.9%), Escherichia (10.8%), *Citrobacter* (8.1%), *Pragia* (6.8%) and *Proteus* (6.8%). Strains of other bacteria could be detected only in one or two samples (Table **1**) only.

From each of the two samples two different strains of the same bacterial species (*Aeromonas veronii*) could be identified using antimicrobial sensitivity pattern (Table **3**). In three of the samples strains of two different species of *Aeromonas* (*A. schubertii+ A. sobria, A. eucranophila + A. salmonicida, A. caviae +A. veronii*) were detected simultaneously. In other three samples strains of two species of *Enterococcus* (*Ec. faecalis and* Ec. *faecium)* were present while in one sample both *Ec. faecalis* and *Ec. casseliflavus* (Table **1**) were detected simultaneously.

Table. 1. Bacteria Isolated from Vaginal Swabs of Pre-Parturient Sows in Nagaland

Number of Bacteria isolated	Total samples (%)	Sample numbers	Types of bacteria isolated
0	18 (24.3)	45, 46, 48, 49, 50, 52, 55, 56, 57, 58, 59, 62, 63, 64, 65, 66, 67, 73	No aerobic or facultative aerobic bacteria could be cultured
1	20 (27.0)	7, 8, 14, 16, 18, 19, 23, 24, 37, 39, 43, 44, 47, 53, 54, 68, 69, 71, 72, 74	*A. caviae* (2), *A. eucranophila* (2), *A. veronii* (2), *C. freundii* (1), *E. fergusonii* (1), *Erwinia ananas* (1), *K. pneumoniae* (6), *Kluyvera crocrescens* (1), *Pragia fontium* (1), *P. vulgaris* (1), *Pseudomonas fluorescens* (2)
2	26 (35.1)	1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 15, 17, 20, 21, 24, 31, 35, 36, 40, 41, 42, 51, 60, 61, 70	*A. caviae + Ec. faecalis* (1), *A. caviae + E. ferfusonii* (1), *A. eucranophila + R. planticola* (1), *A. eucranophila + P. mirabilis* (1), *A. eucranophila + A. salmonicida* (1), *A. eucranophila + Ec. faecalis* (1), *A. hydrophila + Ec. faecium* (1), *A. salmonicida + K. pneumoniae* (1), *A. schubertii + K. oxytoca* (1), *A. sobria + Ec. faecium* (1), *A. veronii + Ec. faecium* (4), *A. veronii + Ec. faecalis* (1), *C. diversus + Ec. faecalis* (1), *C. freundii + Ec. faecalis* (1), *C. freundii + K. pneumoniae* (1), *E. coli + Ec. faecium* (1), *E. coli + Ec. faecalis* (1), *E. fergusonii + Ec. faecalis* (2), *Ec. faecalis + K. pneumoniae* (1), *Ec. faecalis + Leminorella ghrimontii* (1), *Ec. faecium + P. mirabilis* (1), *K. pneumoniae + R. terrigena* (1)
3	5 (6.8)	22, 28, 30, 32, 33	*A. salmonicida+ Ec. faecium+ Streptococcus suis* (1), *A. schubertii+ Ec. faecium+ Pragia fontium* (1), *A. veronii+ Ec. faecium+ K. pneumoniae* (1), *Ec. faecalis+ Ec. faecium+ P. vulgaris* (1), *E. coli+ Ec. faecium+ Edwardsiella tarda* (1)
4	2 (2.7)	27, 29	*A. caviae +A. veronii+ Ec. faecium+ Providencia heimbachae* (1), *A. schubertii+ A. sobria+ Budvicia acquatica+ Ec. faecium* (1)
5	1 (1.4)	34	*A. sobria+ Ec. faecalis+ Ec. faecium+ K. pneumoniae+ Pragia fontium* (1)
6	1 (1.4)	26	*A. hydrophila+ Budvicia aquatica+ C. freundii+ Ec. faecalis+ Ec. faecium+ P. penneri* (1)
8	1 (1.4)	38	*A. veronii+ Ec. faecalis+ Ec. casseliflavus+ C. freundii+ E. fergusonii+ Pragia fontium+ Erwinia ananas+ K. pneumoniae* (1)

Table. 2. Antimicrobial Resistance in Bacteria Isolated from Vaginal Swabs of Pregnant Sows for different Antimicrobials and Herbal Drugs

Bacteria	N	Ca	Cf	Na	C	A	T	G	Co	SWO	AVEO	LGO	PEO	EG	MDR	MHDR
Aeromonas caviae	5	0	0	3	1	5	2	0	3	5	3	0	3	2	3	3
A. eucranophila	6	0	0	5	2	6	2	0	5	3	6	3	4	4	5	4
A. hydrophila	2	0	0	0	0	2	0	0	1	2	1	1	2	2	0	2
A. salmonicida	3	0	0	1	0	3	0	0	0	3	3	2	2	2	0	2
A. schubertii	3	0	0	2	0	3	0	0	0	1	1	0	1	2	0	1
A. sobria	3	0	0	3	1	3	0	0	0	3	1	0	3	3	1	3
A. veronii	10	0	0	6	0	10	1	0	2	10	7	2	10	7	2	10
Budvicia aquatica	2	0	1	2	0	2	0	0	0	2	0	0	2	2	1	2
Citrobacter diversus	1	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1
C. freundii	5	1	0	1	0	2	1	0	1	5	5	2	5	3	0	5
Escherichia coli	3	0	0	0	0	1	1	0	1	3	3	0	3	1	0	3
E. fergusonii	5	0	0	2	1	2	1	0	1	4	5	1	5	3	1	5
Enterococcus casseliflavus	1	0	0	1	0	1	0	0	1	1	1	1	0	1	1	1
Ec. faecalis	15	3	0	15	3	3	2	4	14	15	15	6	9	15	7	15
Ec. faecium	16	1	0	16	0	2	0	0	15	16	16	8	13	16	2	16
Edwardsiella tarda	1	0	0	0	0	0	0	0	1	1	1	0	1	0	0	1
Erwinia ananas	2	0	0	1	0	0	0	0	0	2	2	2	2	2	0	2
Klebsiella oxytoca	1	0	0	1	0	1	0	0	1	0	1	0	0	1	1	0
K. pneumoniae	13	0	0	8	0	13	1	0	3	9	13	6	8	11	2	12
Kluyvera cryocrescens	1	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0
Leminorella ghrimontii	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Pragia fontium	5	0	0	2	1	3	1	0	2	5	5	2	5	4	1	5
Proteus mirabilis	2	0	0	1	1	1	1	0	1	2	2	1	2	1	1	2
Proteus penneri	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1	1
Proteus vulgaris	2	0	0	0	2	0	2	0	2	2	2	1	2	1	2	2
Providencia heimbachae	1	0	0	0	1	1	1	0	0	1	1	0	1	0	1	1
Pseudomonas fluorescence	2	0	0	1	1	2	0	0	1	2	2	2	1	1	1	2
Raoultella planticola	1	0	0	1	0	1	0	0	0	0	1	0	1	1	0	1
Raoultella terrigena	1	0	0	0	0	1	0	0	0	1	1	0	1	1	0	1
Streptococcus suis	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0
Total resistant (%)	115	5* (4.3)	1 (0.9)	75* (65.2)	15 (13.0)	70* (60.9)	17 (14.8)	4* (3.5)	57* (49.6)	100** (87.0)	101*** (87.8)	41 (35.7)	88*** (76.5)	90* (78.3)	33 (28.7)	103** (89.6)
Gram + (%)	33	4 (12.1)	0 (0.0)	33 (100)	3 (9.1)	6 (18.2)	2 (6.1)	4 (12.1)	31 (93.9)	32 (97.0)	32 (97.0)	15 (45.5)	22 (66.7)	33 (100)	10 (30.3)	32 (97.0)
Gram -ve (%)	82	1 (1.2)	1 (1.2)	42 (51.2)	12 (14.6)	64 (78.0)	15 (18.3)	0 (0.0)	26 (31.7)	68 (82.9)	69 (84.1)	26 (31.7)	66 (80.5)	57 (69.5)	23 (28.0)	71 (86.6)

Abbreviations: A, *Aeromonas*; C, *Citrobacter*; E, *Escherichia*; Ec, *Enterococcus*; K, *Klebsiella;* N, numbers tested; Ca, ceftazidime; Cf, ciprofloxacin; Na, nalidixic acid; C, chloramphenicol; A, ampicillin; T, tetracycline; G, gentamicin; Co, cotrimoxazole; SWO, sandalwood oil; AVEO, *Artemesia vulgaris* essential oil; LGO, lemongrass oil; PEO, patchouli essential oil; EG, eucalyptus gum; MDR, multiple drug resistance; MHDR, multiple-herbal-drug-resistance; resistance differed significantly between G+e and G-ve bacteria *(p ≤0.01), **(p 0.01 to 0.05), ***(p >0.05 to 0.1).

Table 3. Antimicrobial Resistance Patterns of Bacteria Isolated from Vaginal Swabs of Pre-Parturient Sows

Antibiogram type	Resistance pattern	No. of strains (%)	Bacteria
AT-1	Sensitive to all antibiotics	8 (7.0)	*E. fergusonii* (2), *Erwinia ananas* (1), *C. freundii* (2), *Leminorella ghrimontii* (1), *E. coli* (1), *Proteus mirabilis* (1)
AT-2	A	18 (15.7)	*A caviae* (1), *A. eucranophila* (1), *A hydrophila* (1), *A. salmonicida* (2), *A. schubertii* (1), *A. veronii* (4), *C. diversus* (1), *E. fergusonii* (1), *K. pneumoniae* (4), *Pragia fontium* (1), *Raoultella terrigena* (1)
AT-3	C	1 (0.9)	*Pragia fontium* (1)
AT-4	Co	2 (1.7)	*Edwardsiella tarda* (1), *Pragia fontium* (1)
AT-5	Na	3 (2.6)	*Erwinia ananas* (1), *E. fergusonii* (1), *Kluyvera cryocrescens* (1)
AT-6	T	1 (0.9)	*E. coli* (1)
AT-7	A, Co	5 (4.3)	*E. coli* (1), *C. freundii* (1), *A. hydrophila* (1), *K. pneumoniae* (1), *Pseudomonas fluorescens* (1)
AT-8	A, Na	20 (17.4)	*A. caviae* (1), *A. salmonicida* (1), *A. schubertii* (2), *A. sobria* (2), *A. veronii* (4), *Budvicia aquatica* (1), *Ec. faecalis* (1), *K. pneumoniae (6)*, *Pragia fontium* (1), *Raoultella terrigena* (1)
AT-9	A, T	1 (0.9)	*C. frendii* (1)
AT-10	A. C, T	1 (0.9)	*Providencia heimbachae* (1)
AT-11	A, C, Na	2 (1.7)	*A. sobria* (1), *Pseudomonas fluorescens* (1)
AT-12	A, Ca, Na	1 (0.9)	*Ec. faecium* (1)
AT-13	A, Cf, Na	1 (0.9)	*Budvicia aquatica* (1)
AT-14	A, Co, Na	8 (7.0)	*A. eucranophila* (2), *A. veronii* (1), *Ec. casseliflavus* (1), *Ec. faecalis* (1), *Ec. faecium* (1), *K. oxytoca* (1), *K. pneumoniae* (1)
AT-15	A, co, T	1 (0.9)	*A. caviae* (1)
AT-16	A,C, Co, Na	2 (1.7)	*A. caviae* (1), *A. eucranophila* (1)
AT-17	A, Co, Na, T	5 (4.3)	*A. caviae* (1), *A. eucranophila* (1), *A. veronii* (1), *K. pneumoniae* (1), *Pragia fontium* (1)
AT-18	A, C, Co, Na, T	4 (3.5)	*A. eucranophila* (1), *E. fergusonii* (1), *Proteus mirabilis* (1), *Proteus penneri* (1)
AT-19	A, Ca, Co, G, Na	1 (0.9)	*Ec. faecalis* (1)
AT-20	Ca, Na	1 (0.9)	*C. freundii* (1)
AT-21	Ca, Co, Na	1 (0.9)	*Ec. faecalis* (1)
AT-22	Ca, Co, G, Na	1 (0.9)	*Ec. faecalis* (1)
AT-23	C, Co, Na	1 (0.9)	*Ec. faecalis* (1)
AT-24	C, Co, G, Na, T	2 (1.7)	*Ec. faecalis* (2)
AT-25	C, Co, T	2 (1.7)	*Proteus vulgaris* (2)
AT-26	Co, Na	22 (19.1)	*Ec. faecalis* (7), *Ec. faecium* (14), *Streptococcus suis* (1)

Abbreviations: A, Aeromonas; C, Citrobacter; E, Escherichia; Ec, Enterococcus; K, Klebsiella

Of the 8 samples positive for *Escherichia* (*E. coli* 3, *E. fergusonii* 5), 6 contained enterococci also. Association (p, 0.022) of *Escherichia* was more common with *Ec. faecalis* (p, 0.027) than with *Ec. faecium* (p, 0.155). However, two samples positive for *E. fergusonii* had no enterococci. Probability of isolation of *Klebseiella pneumoniae* with other bacteria was slightly higher (p, 0.092) than occurrence of the pathogen alone. Though 5 samples each were positive for *Pragia fontium* and *C. freundii*, in pure culture *P. fontium* or *C. freundii* were detected only in one sample each. Samples positive for *Pseudomonas fluorescens* (2) had no other bacteria detectable in vaginal swabs. However, from all the samples positive for *Raoultella* strains other bacteria (*K. neumoniae* or *A. eucranophila*) were also isolated. Co-occurrence of *A. veronii* and *Ec. faecalis* was rare (p, 0.454), but co-occurrence of *A. veronii* and *Ec. faecium* was significantly (p, 0.002) common. Although co-occurrence of most of the aeromonads with enterococci was significantly (p, 0.003) common, *A. eucranophila* was never detected with enterococci.

3.2. Antimicrobial Drug Resistance of Bacterial Isolates

Of the 115 bacteria isolated in the study only eight isolates were sensitive to all the 8 antimicrobials tested (Table **3**). On the basis of antimicrobial drug sensitivity, 115 isolates could be classified into 26 antibiogram types (ATs). Of the 26 ATs, 12 had only one strain each, 5 had two strains each and rest had multiple strains belonging to same or different species (Table **3**). The most common AT was AT-26 (resistant to cotrimoxazole and nalidixic acid, had 22 strains (all belonging to either *Enterococcus* or *Streptococcus* genus), followed by AT-8 (20 strains) and AT-2 (18 strains). A total of 33 bacterial isolates had resistance to three or more drugs and classified as multiple-drug-resistant (MDR). Of the 26 AT types MDR strains were included in 15ATs (AT-10 to AT-19 and AT-21 to AT-25). More than 83% *A. eucranophila* and 60% *A. caviae* were MDR type with much higher probability than other aeromonads and isolates of other genera in the study (P, 0.001). Of the five isolates of *Proteus* spp. four had MDR with comparatively higher probability (P, 0.01) than other bacteria in pig vagina.

All GPBs and 51.2% of GNBs were resistant to nalidixic acid (p, <0.01). Next commonly resisted drug was ampicillin for that most of the GNBs were resistant (75.6%) but only few GPBs (18.2%) had resistance to it. The next less effective antimicrobial drug was co-trimoxazole (50%), almost all GPBs (93.9%) and 31.7% GNBs were resistant to it. Ciprofloxacin was the most effective antibiotic inhibiting 99.2% isolates; only one isolate (*Budvicia aquatica*) was resistant to ciprofloxacin. Next in effectiveness were gentamicin (96.5%) and ceftriaxone (95.7%), for both the antibiotics GPBs were significantly more resistant (p, <0.01) than GNBs. In contrast, most of the isolates resistant to tetracycline (p, 0.01) belonged to GNBs (15 of the 17 resistant).

All 32 aeromonads and 14 klebsiellae were resistant to ampicillin but had more probability of being sensitive to co-trimoxazole (p, 0.04) than other microbes in the study (Table 2). In contrast, enterococci were more often sensitive to ampicillin (p, 0.001) and resistant to co-trimoxazole (p, 0.001). Besides, enterococci were more commonly resistant to ceftazidime (p, 0.008), nalidixic acid acid (p, 0.0001) and gentamicin (p, 0.001) than other bacteria (Table **2**).

Sensitivity assay to GPB specific antibiotics indicated that resistance was observed only in enterococci but not in *S. suis* isolate. None of the enterococci isolate was resistant to vancomycin. However, more than two third (68.8%) isolates were resistant to oxacillin, and 56.3% to erythromycin and clindamycin each.

3.3. Herbal Antimicrobial Drug Resistance in Bacterial Isolates

Resistance against herbal antimicrobials was very common and detected in 87.8%, 87%, 78.3%, 76.5% and 35.7% isolates against AVEO, SWO, EG, PEO and LGO, respectively. Except for LGO, resistance to other herbal antimicrobials was more common in sow vaginal isolates of aerobic bacteria than nalidixic acid (65.2%) and ampicillin (60.9%), the least effective antibiotics. Though sensitivity of GPBs (enterococci) was less frequent than GNBs for SWO (p, 0.04), AVEO (P, 0.06) and EG (P, 0.003), no difference was evident towards sensitivity to LGO and PEO. Among GNBs, aeromonads had fewer chances to be resistant to AVEO (P, 0.001) but no significant difference was evident for other herbal drugs.

Multiple herbal antimicrobial resistance (MHAR) was detected in 89.6% strains tested. The MHAR was almost three times more common than MDR (28.7%) with high probability (P, 0.02) in enterococci (GPBs). Among GNBs, members of Enterobacteriaceae (92 %) were the more frequent carriers (P, 0.07) of MHAR than aeromonads (78.1%).

4. DISCUSSION

Although in most of the studies anaerobic bacteria are found to outnumber aerobic bacteria in vagina but aerobically growing microbes have been shown to be some times more important because of their ability anaerobically (facultative anaerobes) and ability to cause infections [8]. Moreover in sows and other animals facultative anaerobes (able to grow both aerobically and anaerobically) have been reported to cause major shift in reproductive health of mothers and postnatal health of infants [14, 24]. Thus the study of aerobic microbiome of vagina is as important as anaerobic microbiome. However, for complete understanding all microbes including non-culturable ones should be analysed but due to limited microbiological facilities more important aspect was taken into consideration.

The study on mesophilic aerobic microbiome of 74 pre-parturient sow vaginal swabs, no bacteria could be isolated from 18 (24.3%) vaginal swabs of sows. In 20

(27%) sample only one type while in 36 (48.7%) sample more than one type of bacteria were detected (Table **1**). Bacteria identified from sow vaginal swabs belonged to 30 different species (Table **2**). Absence of any aerobically growing bacteria in vagina of sows may not be surprising because vaginal flora is mainly constituted by anaerobes [7, 8]. Detection of aerobically growing bacteria from vaginal swabs of >75% sows indicated importance of aerobes in constitution of microbiome of sow vagina.

The isolates belonged to as many as 26 antibiogram types (Table **3**). Similar type of microbial diversity has been reported earlier in vagina of healthy as well as with reproductive tract infections in sows [14, 22-24] and in vagina of other animals [9-11, 13, 15-18] too. Understanding presence of potentially pathogenic facultative anaerobic bacteria in vagina of pre-parturient sows may be significant in planning management strategies to enhance the production in light of facts that vaginal secretions were the most likely source of infection for piglets [30].

Bacterial isolates from sow vagina belonged to three major groups of facultatively anaerobic bacteria including members of Enterobacteriaceae (50), *Aeromonas* spp. (32) and *Enterococcus* spp. (32) and none of the isolate was strict aerobe. In earlier studies on sows and feral sows in different parts of world enterococci and enterobacteria have been reported as the most common urogenital commensal as well as opportunistic pathogens associated with uro-genital infections [22, 23, 31]. Although, aeromonads have rarely been reported from sow vagina [23], in the same region (Jharnapani, Nagaland) strains belonging to similar species of aeromonads have commonly been reported from rectum of diarrhoeic pigs [32] and vagina of swamp buffaloes [15] and mithuns [16]. In the earlier study on same farm [32] many of the bacteria isolated from vagina of sows were identified as cause of diarrhoea in piglets indicating dynamism in movement pathogens.

Though enterobacteria were detected in 50% of sow vagina, *E. coli* was isolated only from three sows. In earlier studies on microflora of sow reproductive tract *E. coli* have been reported to constitute a major group [22, 23, 33]. On the same farm in earlier studies on diarrhoeic piglets, *E. coli* was identified as the most prominent (26 of 36) cause of disease [32]. The less frequency of occurrence of *E. coli* in pre-parturient sows might be due to prevalence of mucosal immunity in the farm as observed in case of application of streptococci vaccine [30]. The other reason might be an alternate source of diarrhoeagenic *E. coli* in piggeries as suspected in earlier report [32].

Besides enterococci, *S. suis* could be detected in a sow vagina. *Streptococcus suis* is an important pathogen of swine and reported in their reproductive tracts [22]. Birth canal (cervix and vagina) has been found to be the most common source of the infection in piglets [19].

Some of the potentially pathogenic bacteria isolated from sow vaginal swabs including *Edwardsiella tarda, Raoultella* spp. and *Klebsiella oxytoca*, have been rarely reported earlier from reproductive tract of pigs [14, 21-23]. However, these microbes may cause serious illness in pigs and *E. tarda* has been often associated with diarrhoea in piglets [32, 34, 35]. On the other hand, *K. pneumoniae* isolated from 13 vaginal swabs of sows has been commonly reported pathogen of pigs present in reproductive tract, respiratory as well as in genital tract [20, 23, 36, 37].

Several bacteria often isolated from environment including strains of *Pseudomonas, Citrobacter, Erwinia, Proteus, Kluyvera, Pragia* and *Leminorella* species were also isolated from sow vaginal swabs. Many of them have also been reported to be present in reproductive tract of sows and other animals [9, 10, 17, 18, 23, 33, 38] and a few of them have also been associated with reproductive tract infection in sows too [20, 22, 23].

Extensive antimicrobial resistance and MDR is now common everywhere irrespective of source of microbes [39]. Therefore isolation of large number of MDR bacteria from vagina of sow is neither novel nor unexpected, similar antimicrobial drug ressitance has been reported earlier in bacteria isolated from the same piggery [32], from feral pigs in Brazil [23] and even from vaginal swabs of other animals [15, 16] and environmental sources in the same region Jharnapani [40] and also in other parts of India [9].

On the basis of antibiogram isolates could be classified into 26 types. The isolates of the same species could easily be differentiated from one other to identify them into different strains (Table **3**). Observations revealed the diversity of microflora in vagina even on the same farm indicating that there is some individuality in microbiome of vagina of sows of the same species at the same farm under similar husbandry conditions as observed in human beings and other life forms [41].

Resistance of all aeromonads and klebsiellae to ampicillin observed the study is not alarming because these potential pathogens are usually resistant to ampicillin [23, 42].

Enterococci isolates from sow vagina were mostly sensitive to ampicillin and resistant to co-trimoxazole. In earlier studies on antimicrobial sensitivity of vaginal microbiome of feral pigs [23] and of other animals [9, 10, 15, 16, 43] enterococci have been reported to be sensitive to ampicillin and amoxicillin but resistance to cotrimoxazole. In earlier studies vancomycin resistant isolates of enterococci has been reported from vagina of pigs [23] and mares [9, 10, 43] but in the present study none of enterococci isolates had resistance to vancomycin. It might be due non-use of the drug in Jharnapani and nearby and similar observations were made earlier on enterococci isolates from mithun [16] and swamp-buffalo [15] vagina in Nagaland and also from diarrhoeic piglets of the same farm (32). However, 68.8% and 56.3% isolates were resistant to oxacillin, and erythromycin (56.3%). Oxacillin and erthryomycin resistance though more important for staphylococci [28] has commonly been reported earlier in enterococci of Jharnapani region from different sources [15, 16, 32] and indicated that it might have spread to different microbiomes irrespective of animal and their systems.

Although defining herbal drug resistance is always been a controversial issue due to use of different concentrations of herbs in discs used, one has to set some cut off limit [29], in our study we uniformly used 1 mg of herbal preparation (either pure oil or dried extract). The bacteria inhibited to grow around disc were classified resistant as reported in several earlier studies [29, 40]. Resistance to AVEO, SWO, EG, PEO was observed in more isolates than resistance to any of the least effective antimicrobials. It is not much different from herbal drug resistance reported in semi-domestic animals of the same locality (Swamp buffaloes, Mithuns) or in domiciled birds and lizards [15, 16, 29, 32, 44]. In semidomestic and feral animals herbal drug resistance in Nagaland might expected due to continuous exposure of those animals to many of the herbs growing wild in Nagaland. However presence of herbal drug resistance in farmed animals indicated that microflora in different microbiomes is under dynamic state and similar to antibiotic resistance [28, 39] herbal drug resistance is also spreadable. Lemmon grass oil inhibited growth of almost two third (64.3%) of strains and might be promising natural antimicrobial in control of infections and feed additives. The observations on affectivity of LGO on microbes are in concurrence to earlier studies in the same region on microbes of environmental and other animal origin [44].

5. SUMMARY

The study indicated that aerobically culturable microbiome which is constituted exclusively by facultative anaerobe might be important due to their ability as opportunistic pathogens and ability to grow and compete with anaerobic flora of healthy vagina. The study revealed that in pre-parturient sow vagina the aerobically culturable microbiome is diverse and many potentially pathogenic bacteria may be its constituents. The information may be vital in planning for better management of piggery and can also be an indicator of health. Besides, understanding normal microbiome of sow vagina, it may help in understanding the reality of alarms and can be interpreted to avoid the un-necessary worries associated with detection of potentially pathogenic bacteria during routine screening. The study also indicated individuality in microbiome as well as spread of antimicrobial resistance in bacteria.

ACKNOWLEDGEMENTS

Authors are thankful to the Director ICARRC for NEH Region and Joint Director of Nagaland Centre for supporting the studies and Mis. Moa for technical assistance in laboratory.

REFERENCES

[1] Strömbeck L. Vaginal commensal bacteria: Interactions with cervix epithelial and monocytic cells and influence on cytokines and secretory leukocyte protease inhibitor, SLPI. PhD thesis Gothenburg University, Sweden 2008.

[2] Rose WA II, McGowin CL, Spagnuolo RA, Eaves-Pyles TD, Popov VL, Pyles RB Commensal Bacteria Modulate Innate Immune Responses of Vaginal Epithelial Cell Multilayer Cultures. PLoS ONE 2012; 7: e32728. http://dx.doi.org/10.1371/journal.pone.0032728

[3] Srinivasan S, Fredricks DN The human vaginal bacterial biota and bacterial vaginosis. Interdisciplinary Perspectives on Infectious Diseases. doi:10.1155/2008/750479, 2008, pp. 22.

[4] Cho CE, Norman M. Cesarean section and development of the immune system in the offspring. Am J Obstet Gynaecol 2013; 208: 249-54. http://dx.doi.org/10.1016/j.ajog.2012.08.009

[5] Hansen CHF, Andersen LSF, Krych L, Metzdorff SB, Hasselby JP, Skov S, Nielsen DS, Buschard K, Hansen LH, Hansen AK. Mode of Delivery Shapes Gut Colonization Pattern and Modulates Regulatory Immunity in Mice. J Immunol 2014; 193: 973-4. http://dx.doi.org/10.4049/jimmunol.1400085

[6] Singh BR, Singh V, Ebibeni N, Singh RK. Maternal Transfer of Bacteria to Eggs of Common House Gecko (Hemidactylus frenatus). J Microbiol Res 2014; 4:78-85.

[7] Larsen B, Galask R. Vaginal microbial flora: composition and influence of host physiology. Ann Internal Med 1982; 96: 926-30. http://dx.doi.org/10.7326/0003-4819-96-6-926

[8] Larsen B, Gilles MRG. Understanding the bacterial flora of the female genital tract. Clin Infect Dis 2001; 32: e69-77. http://dx.doi.org/10.1086/318710

[9] Singh BR. Occurrence of multiple drug resistant (MDR) aerobic bacteria in vaginal swabs of mares and their association with infertility. Indian J Comp Microbiol Immunol Infect Dis 2010; 30: 105-12.

[10] Singh BR. Thermotolerance and multidrug resistance in bacteria isolated from equids and their environment. Vet Rec 2009; 164:746-50. http://dx.doi.org/10.1136/vr.164.24.746

[11] Chotimanukul S, Sirivaidyapong S. The Relationship of Canine Vaginal and Uterine Bacterial Species in Closed-Cervix and Opened-Cervix Pyometra. In: Proceedings of 13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Bangkok,Thailand 2010; pp. 184-6.

[12] Lynch CD, Iams JD. Diseases resulting from suboptimal immune function in offspring: is cesarean delivery itself really to blame? Am J Obstet Gynecol 2013; 208: 247-248. http://dx.doi.org/10.1016/j.ajog.2012.12.027

[13] Mshelia GD, Amin, Chaudhary SUR. Vaginal Bacterial Flora of Nigeria Local Bitches During Different Stages of Reproductive Cycle. Int J Agric Biol 2001; 3: 183-5.

[14] Bara MR, McGowan MR, O'Boyle D, Cameron RD. A study of the microbial flora of the anterior vagina of normal sows during different stages of the reproductive cycle. Aust Vet J 1993; 70: 256-9. http://dx.doi.org/10.1111/j.1751-0813.1993.tb08043.x

[15] Singh BR, Singh RK, Singh V. Carriage of Multiple drug Resistant Bacteria in Vagina of Apparently Healthy Swamp Buffaloes in Nagaland. Ad Anim Vet Sci 2014; 2: 292-5. http://dx.doi.org/10.14737/journal.aavs/2014/2.5.292.295

[16] Singh BR, Singh RK, Singh V. Aerobic mesophilic bacterial flora of vagina of healthy Mithun (Bos frontalis). J Genital Sys Disord 2015; 3: 1-7.

[17] Hafner LM, Rush CM, Timms P. The Vaginal Microbiota of Guinea Pigs. Microb Ecol Hlth Dis 2009; 9: 123-7. http://dx.doi.org/10.3109/08910609609166452

[18] Zhi LB, Jun ZX, Yu MZ, Qing ZK. Difference of aerobic and facultative anaerobic bacteria in vagina of postpartum healthy cows and endometritis cows. J Northwest Agric Forest Uni, 2008; 11: 57-60.

[19] Robertson ID, Blackmore DK. Prevalence of Streptococcus suis types 1 and 2 in domestic piglets in Australia and New Zealand. Vet Rec 1989; 124:391-4. http://dx.doi.org/10.1136/vr.124.15.391

[20] Xaver T, Glock P, Bilkei G. The effect of postparturient urogenital diseases on the lifetime reproductive performance of sows. Canad Vet J 2005; 46: 1103-7.

[21] Ungerfeld R, Silva L. Presence of normal vaginal flora is necessary for normal sexual attractiveness of estrous ewes. Appl Anim Behav Sci 2005; 93: 245-50. http://dx.doi.org/10.1016/j.applanim.2004.11.014

[22] Vlcek Z, Svobodova R. The Dynamics of Bacterial Contamination of the Genital Organs in Sows During the Puerperium. Acta Vet Brno 1980; 49: 231-6. http://dx.doi.org/10.2754/avb198049030231

[23] Lessa SS, Paes RCS, Santoro PN, Mauro RA, Vieira-da-Motta O. Identification and antimicrobial resistance of microflora colonizing feral pig (Sus scrofa) of Brazilian pantanal. . Braz J Microbiol 2011; 42: 740-9. http://dx.doi.org/10.1590/S1517-83822011000200042

[24] De Winter PJJ, Verdonck M, de Kruif A, Devriese LA, Haesebrouck F. Bacterial endometritis and vaginal discharge in the sow: prevalence of different bacterial species and experimental reproduction of the syndrome. Anim Reprod Sci 1995; 37: 325-335. http://dx.doi.org/10.1016/0378-4320(94)01342-J

[25] Patra MK, Begum S, Deka BC. Problems and Prospects of Traditional Pig Farming for Tribal Livelihood in Nagaland. Indian Res J Ext Edu 2014; 14(s): 17-21.

[26] Carter GR. Diagnostic Procedures in Veterinary Microbiology, 2nd ed. Charles C Thomas Publishers; Springfield 1975.

[27] Singh BR. Labtop for Microbiology Laboratory. Lambert Academic Publishing; Germany 2009.

[28] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. 24th informational supplement, Document M100-S24 and M11-A8. Clinical and Laboratory Standards Institute; Wayne, Pennsylvania 2014.

[29] Singh BR. Evaluation of antibacterial activity of Salvia officinalis [L.] sage oil on veterinary clinical isolates of bacteria. Noto-are: Med 2013. https://www.notoare.com/index.php/index/explorer/getPDF/15341289

[30] Woods RD, Ross FR. Immunogenicity of experimental Streptococcus equisimilis vaccines in swine. Am J Vet Res 1977; 38: 33-6.

[31] Colman J, Devriese L, Verdonck M. Bacteruria and urinary tract infection in sows. Vlaams Diergeneesk Tijdschr, 1988; 57: 192-8.

[32] Singh BR. Drug Resistance in Enteropathogens of Zoonotic Significance Isolated from Diarrhoeic Pigs and House Sparrow Perching in Piggery in Jharnapani, Nagaland. Noto-are Med 2012; URL: https://www.notoare.com/ 17378234.

[33] Maes D, Verdonck M, De Kruif A. Vaginal microecology and vulval discharge in swine. In: Peter J Heidt, Philip B Carter, Volker R, Dirk van der Waaij eds. Old Herborn University Seminar Monograph 12: Vaginal flora in health and disease. Herborn-Dill, Germany: Herborn Litterae 1999; pp. 39-50.

[34] Singh BR, Tiwari AK. Toxigenicity of Edwardsiella tarda isolates of fish and pig origin in experimental models. Indian J Exp Biol 1996; 34: 1254-6.

[35] Singh BR. Multiple Drug Resistant Edwardsiella tarda and Edwardsiella hoshinae in an Integrated Fish-pig-duck Farm and Down Colony in Jharnapani, Nagaland. Noto-are: Med 2014 URL: https://www.notoare.com/index.php/index/explorer/getPDF/12446517.

[36] Singh BR, Sharma VD, Chandra R. A study on virulence markers of Klebsiella isolated from foods and clinical samples. J Food Sci Technol 2000; 37: 348-56.

[37] Singh BR, Sharma VD. Haemagglutination pattern of Klebsiella, their pathogenic potential and intestinal colonization. J Food Sci Technol 2000; 37: 400-5.

[38] Wegener HC, Skov-Jensen EW. A longitudinal study of Staphylococcus hyicus colonization of vagina of gilts and trans-mission to piglets. Epidemiol Infect 1992; 109: 433-44. http://dx.doi.org/10.1017/S0950268800050421

[39] Kumar S, Singh BR. An Overview on Mechanisms and Emergence of Antimicrobials Drug Resistance. Ad Anim Vet Sci 2013; 1 (2S): 7-14.

[40] Singh BR, Singh V, Ebibeni N, Singh RK. Antimicrobial and herbal drug resistance in enteric bacteria isolated from faecal droppings of common house lizard/gecko (Hemidactylus frenatus). Int J Microbiol 2013; doi:10.1155/2013/340848.

[41] Sunagawa S, Schloissnig S, Arumugam M, Forslund K, Mitreva M, Tap J, Zhu A, Waller A, Mende DR., Kultima JR., Martin J, Kota K, Sunyaev SR, Typas A, Weinstock GM, Bork P. Individuality and temporal stability of the human gut microbiome. Central Asian J Glob Hlth, 2013; 2S: doi 10.5195/cajgh.2013.120. http://dx.doi.org/10.5195/cajgh.2013.120

[42] Singh BR, Kulshreshtha SB. Isolation of Ampicillin sensitive Aeromonas from aquatic foods with XLD Agar and ampicillin dextrin agar. J Food Sci Technol 1993; 30: 359-61.

[43] Singh BR. Prevalence of vancomycin resistance and multiple drug resistance in enterococci in equids in North India. J Infect Develop Cties 2009; 3: 498-503. http://dx.doi.org/10.3855/jidc.467

[44] Singh BR, Singh V, Singh RK, Ebibeni N. Antimicrobial activity of lemongrass (Cymbopogon citratus) oil against microbes of environmental, clinical and food origin. Int Res J Pharm Pharmacol 2011; 1: 228-36.

Received on 12-10-2015 Accepted on 02-02-2016 Published on 09-05-2016

DOI: http://dx.doi.org/10.20941/2310-8703.2016.04.2