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Isolation and Characterization of Antibiotic Resistant Bacteria from Swiftlet Feces in Swiftlet Farm Houses in Sarawak, Malaysia

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There is a growing concern on the occurrence of antimicrobial resistance. Development of multiple antibiotic resistant bacteria has overtaken new drug development and threatened the patients with untreatable infections. This study was conducted to isolate and characterize the antibiotic resistant bacteria from swiftlet farm houses located in various places including Kota Samarahan, Semarang, Saratok, Betong, Sarikei, Sibu, Sepinang, Maludam, Miri, and Kuching in Sarawak, Malaysia. Five feces samples were collected randomly from each site. One gram of the feces sample was diluted in 9 mL of 0.85% normal saline solution. The diluted sample was plated on Trypticase Soy agar plates and incubated at 37±1 °C for 24 h. A total of 500 bacteria isolates were then identified using 16S rRNA analysis method. Disc diffusion method was then used to confirm the resistant phenotypes of these isolates. The results showed that the means of the bacterial colony count were significantly different ($p<0.05$) from one another, with the highest $\log_{10}$ CFU g^{-1} (9.22±0.72) found in Kota Samarahan and the lowest $\log_{10}$ CFU g^{-1} (6.03±0.62) in Betong. Besides, the isolated bacteria were identified as 96% Gram positive bacteria and 4% Gram negative bacteria. The isolated bacteria were highly resistant to penicillin G (36.80±23.87%), ampicillin (28.60±17.13%), and rifampicin (16.90±13.70%). Thus, swiftlet feces are good reservoir for a range of antibiotic resistant bacteria which may pose a potential health hazard to human.

Key words: antibiotic resistance, bacteria, isolation, swiftlet feces

Dewasa ini, tingkat kekhawatiran pada munculnya resistensi terhadap antimikroba semakin meningkat. Laju kemunculan bakteri dengan resistensi ganda terhadap antibiotik lebih tinggi dibandingkan dengan penemuan obat baru, sehingga membahayakan pasien-pasien dengan infeksi yang tidak tertangani. Penelitian ini ditujukan untuk mengisolasi bakteri yang resisten terhadap antibiotik dari peternakan walet (swiftlet) yang tersebar di Kota Samarahan, Semarang, Saratok, Betong, Sarikei, Sibu, Sepinang, Maludam, Miri, dan Kuching di Sarawak, Malaysia dan mengkarakterisasinya. Lima sampel feses diambil secara acak dari masing-masing situs. Satu gram sampel fees didilusi dalam 9 mL 0.85% larutan salin normal. Sample terdilusi kemudian di sebar pada media agar *Tripticase Soy* dan diinkubasi pada 37±1 °C selama 24 jam. Lima ratus isolat bakteria kemudian diidentifikasi berdasarkan urutan 16S rRNA. Resistensi dibuktikan menggunakan metode cakram difusi (*disk diffusion method*). Hasil penelitian ini menunjukkan bahwa nilai rata-rata penghitungan koloni bakteri berbeda secara signifikan ($p<0.05$) antara isolat satu dengan lainnya. Nilai $\log_{10}$ CFU g^{-1} tertinggi ditemukan di Kota Samarahan (9.22±0.72) sementara nilai terendah ditemukan di Betong (6.03±0.62). Identifikasi juga menunjukkan bahwa 96% isolat adalah bakteri gram positif sementara hanya 4% bakteri gram negatif. Bakteri terisolasi menunjukkan resistensi tinggi terhadap Penisilin G (36.80±23.87%), Ampisilin (28.60±17.13%), dan Rifampisin (16.90±13.70%). Maka dapat disimpulkan bahwa feses walet merupakan sumber bakteri yang resisten antibiotik, sehingga boleh jadi berpotensi berbahaya terhadap manusia.

Kata kuci: bakteri,feses walet, isolasi, resistensi antibiotik

Abuse of antibiotic use is becoming a major concern of today's society. It is also the major cause of antibiotic resistance in clinical practice. Since the discovery of penicillin in the 1940s, the emergence of antibiotic resistance has been highlighted. According to Dorsch (2007), the excessive use of antibacterials in agriculture and clinical therapy has led to the phenomenon of antibiotic resistance. Antibiotic resistance decreases our ability in treating infections and diseases, affecting the proper infection control, and prevention strategies (Dorsch, 2007). Antibiotic resistant bacteria have also widely spread in the environment. Transmission of resistant gene in these pathogenic bacteria may cause health problem to human. According to Varaldo (2002), *Streptococcus pyogenes* has become resistant to erythromycin and able to enter human respiratory cells. *Staphylococcus aureus* has also been found to be resistant to a variety of antibiotics (Akano *et al*. 2009). Thus, the studies of

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antibiotic resistance are as vital as providing optimum treatment for patients (Dorsch 2007) and predicting the emerging of resistant pathogens (Allen *et al*. 2010).

Swiftlet feces are usually used as organic fertilizer in agricultural industry because it is rich in nutrient, water, nitrogen, phosphorus, potassium, and minerals (Nyakundi and Mwangi 2011). Most bacteria are able to grow in the swiftlet feces. Organic fertilizers containing these antibiotic resistant bacteria may contaminate vegetables. Improper washing of the contaminated vegetables may also spoil our foods in the kitchen, causing harmful diseases to human. Intensive and extensive abuse of antibiotics usage have caused the formation of an antibiotic resistant genes pool in the environment. Thus, there is a growing public health concern on the microbiological risk of antibiotic resistance of these bacteria in the swiftlet feces to human health. Therefore, this study was conducted to identify and characterize the antibiotic resistant bacteria present in the swiftlet farm houses.

MATERIALS AND METHODS

Locations of the Study Areas. Swiftlet houses were situated at Kota Samarahan, Semarang, Saratok, Betong, Sarikei, Sibu, Sepinang, Maludam, Miri, and Kuching in Sarawak, Malaysia.

Sample Collection. Sampling and sample processing procedures for the isolation of bacteria from the swiftlet feces were carried out as described by Nyakundi and Mwangi (2011). Five samples were collected randomly from the floor of the sampling sites in each swiftlet house. The samples were collected using the spatula and then transferred into sterile bag for storage and then further analysed in the laboratory. Sample was diluted by mixing 1 g of dropping in 9 mL of sterile 0.85% saline solutio\n. The diluted sample was plated on Trypticase Soy agar (Scharlau, Spain) plates and incubated at 37±1 °C for 24 h. The number of bacterial colony forming unit (CFU g^{-1}) was calculated according to Nyakundi and Mwangi (2011) as the number of colony per plate multiplied by the dilution factor. Five to 10 colonies were randomly picked for further identification.

DNA Extraction. The bacterial DNA was extracted by boiling method described by Maria *et al.* (2008) with minor modification. The bacterial cultures were prepared by growing the bacteria in Luria broth (Scharlau, Spain) at 37 ± 1°C for 24 h. One thousand and 500 µL of overnight bacterial culture was transferred into 1.5 mL microcentrifuge tube and centrifuged at 10 000 rpm for 5 min. The supernatant was then discarded. Five hundred microliters of sterile distilled water was added and vortexed to resuspend the cell pellet. The content of the microcentrifuge tubes were then boiled for 10 min together with the tubes and then immediately placed on ice for 5 min. After 5 min, the microcentrifuge tubes were centrifuged at 10 000 rpm for 10 min and the supernatant was collected.

DNA Sequencing Polymerase Chain Reaction Analysis. The isolated bacteria were genotypically characterized using 16S rRNA identification method with minor modification (Woo *et al*. 2000; Kumar *et al.* 2009). Primers 27F (5'-AGAGTTTGATC CTGGCTCA G-3') and 519R (5'-GWATTACCGCGG CKGCTG-3') were used. PCR was performed in reaction mixtures containing 20 µL of DNA, 1.0 µL of each 20 pmol primers (First Base), 3 µL of 10 mM deoxynucleoside triphosphates mix (Promega), 6.0 µL of 25 mM $MgCl_2$ (Promega), 10.0 µL of 5x Buffer solution (Promega), 8.0µL of distilled water, and 1µL of *Taq* polymerase (Promega). PCR was performed for 35 cycles with initial denaturation at 95 °C for 10 min, denaturation at 94 °C for 30 sec, annealing at 55 °C for 1 min, primer extension 72 °C for 1.5 min, and final extension at 72 °C for 10 min. The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis for 30 min at 90 volt.

Sequencing Analysis. DNA purification was carried out using QIAGEN sequencing purifying kit (QIAquick Gel Extraction Kit, USA). Purified DNA product was sent for DNA sequencing at First BASE Laboratories Sdn. Bhd. in Selangor, Malaysia. All the DNA sequences were compared using BLAST program to determine the closest matches of the identities.

Disc Diffusion Method. Antibiotic susceptibility testing was conducted using disc diffusion method according to the method described by National Committee for Clinical Laboratory Standards (NCCLS) (2012) and Zulkifli *et al*. (2009). Antibiotic discs (Oxoid, England) used for testing represents agents historically or currently used in clinical practice.

Escherichia coli ATCC 25922 was used as positive control. Among the antibiotic discs used in this testing were chloramphenicol (30 µg), ampicillin (10 µg), tetracycline (30 µg), streptomycin (10 µg), gentamycin (10 µg), erythromycin (15 µg), cephalothin (30 µg), nitrofurantoin (300 µg), tobramycin (10 µg), rifampicin (5µg), kanamycin (30 g), sulphamethoxazole/trimethoprim (1.25/ 23.75 µg), amikacin (30 µg), imipenem (10 µg), ceftriaxone (30 µg), penicillin G (10 U), doxycycline (30 µg), ceftazidime (30 µg),

norflaxacin (10 µg), vancomycin (30 µg), piperacillin (100 µg), Ciprofloxacin (5 µg), and Nalidixic acid (30 µg). The medium used in this study was Mueller-Hinton agar (MHA). A sterile cotton swab was dipped into a bacterial suspension with turbidity 0.5 McFarland and used to inoculate the surface of the MHA plate evenly, using different bacteria for each plate. The plate was allowed to dry for 5 min. A pair of sterile forceps was used to pick up the antibiotic disc and placed on the surface of the MHA agar plate. The plate was incubated at 37±1 °C for 24 h. The diameter of inhibition zone was measured and the results were recorded as sensitive (S) or resistance (R) base on WHO Drug Information and NCCLS (Yah *et al*. 2007).

Statistical Analysis. Statistical analysis of the data was conducted using SPSS Statistics version 21.0 program. Besides, all the data were analyzed for analysis of variance (ANOVA). All differences between the means were compared using Turkey multiple range test after a significant *F*-test at P<0.05.

RESULTS

Bacterial Colony Count. Table 1 showed that the means of the bacterial colony counts ($\log_{10}$ CFU g^{-1}) were significantly different (F=8.265, P<0.05) from one sampling site to another The highest $\log_{10}$ CFU g^{-1} of 9.22±0.72 was found in Kota Samarahan and the lowest $\log_{10}$ CFU g^{-1} of 6.03±0.62 in Betong. The mean bacterial colony counts collected from Kota Samarahan, Maludam, Miri, Kuching, and Sibu were significantly higher than those from other sampling sites such as Saratok, Betong, Semarang, Sepinang, and Sarikei.

Identification of Bacteria in Swiftlet Feces. A total of 500 bacterial isolates were obtained from Kota Samarahan, Saratok, Betong, Maludam, Miri, Kuching, Semarang, Sepinang, Sarikei, and Sibu. The results showed that a total of 480 (96%) bacterial isolates were identified as Gram positive, while only 20 (4%) were identified as Gram negative bacteria. The distribution of the bacterial isolates in the swiftlet feces from the different sampling sites is shown in Fig 1. Although all these bacterial isolates were found in all the sites, *Staphylococcus* sp. was the most highly prevalent bacteria, followed by *Bacillus* sp., *Lysinibacillus* sp., *Enterococcus harae*, *Sporosarcina* sp., *E. coli*, *Paenibacillus* sp., *Pseudomonas aeruginosa*, *Leucobac-ter iarius*, and *Dermacoccus* sp.

Antibiotic Susceptibility Test. A total of 500 bacterial isolates were identified and disc diffusion method was then used to confirm the resistant phenotypes of these isolates. The percentages of resistance to 23 types of antibiotics among bacteria isolated from Kota Samarahan, Saratok, Betong, Maludam, Miri, Kuching, Semarang, Sepinang, Sarikei, and Sibu was shown in Tables 2. The highest frequencies of antibiotic resistance were detected against penicillin G (36.80±23.87%), followed by ampicillin (28.60±17.13%), and rifampicin (16.90±13.

Table 1 The means of colony counts of bacteria isolated from Kota Samarahan, Saratok, Betong, Maludam, Miri, Kuching, Semarang, Sepinang, Sarikei, and Sibu in Sarawak, Malaysia

Site	Mean of bacterial colony counts ($\log_{10}$ CFU g^{-1})
Kota Samarahan	9.22 ± 0.72 ^{a*}
Saratok	6.23 ± 0.93 ^{b}
Betong	6.03 ± 0.62 ^{b}
Maludam	7.47 ± 0.49 ^{abcd}
Miri	8.76 ± 0.14 ^{ad}
Kuching	8.38 ± 0.16 ^{acd}
Semarang	7.14 ± 0.50 ^{bcd}
Sepinang	6.64 ± 0.83 ^{bc}
Sarikei	6.95 ± 1.68 ^{bc}
Sibu	7.52 ± 1.01 ^{abcd}
Total	7.43 ± 1.26

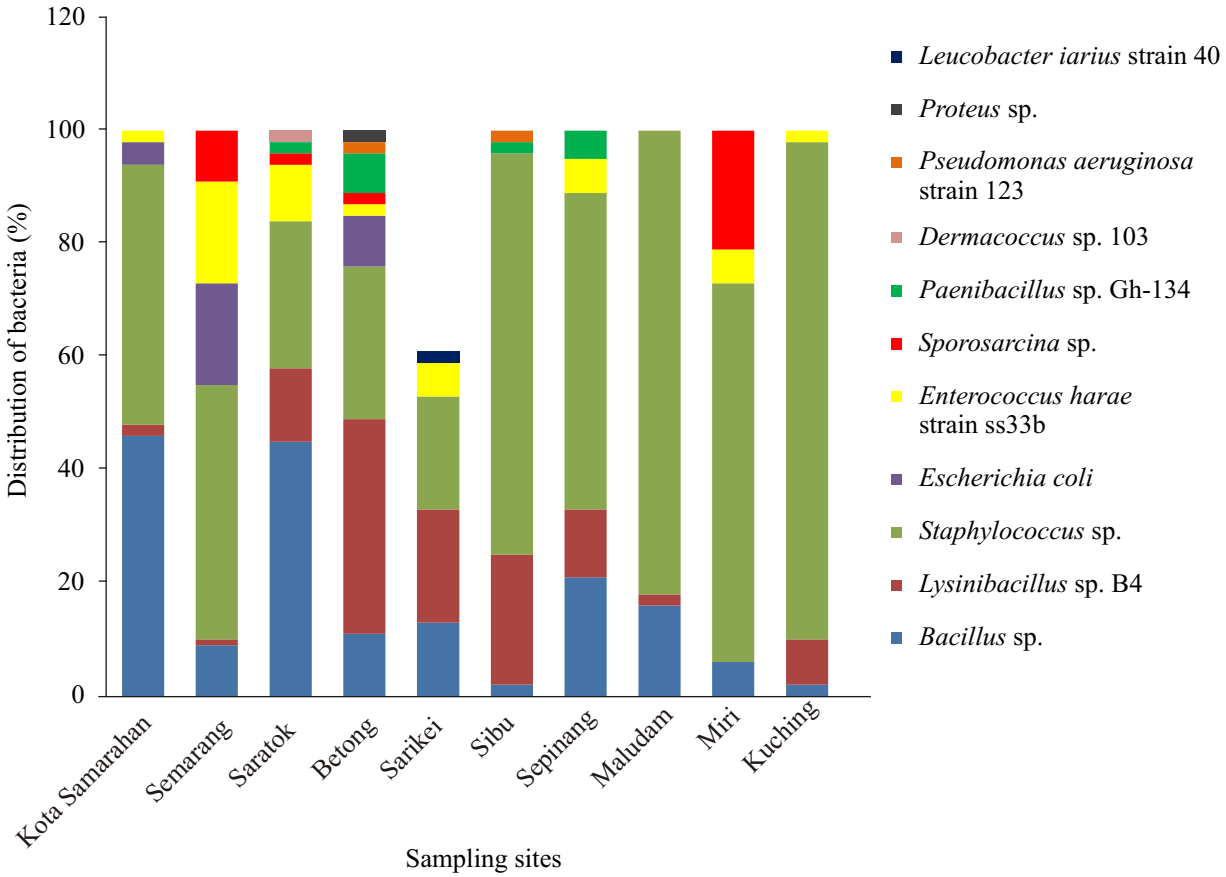

Fig 1 Swiftlet feces bacterial distributions (%) in Kota Samarahan, Semarang, Saratok, Betong, Sarikei, Sibu, Sepinang, Maludam, Miri, and Kuching in Sarawak, Malaysia.

70%). The bacteria isolated from different sites showed different levels of resistance toward the antibiotics (Table 2). The bacteria have developed resistance to at least one antibiotic. The analysis of variance test showed that the levels of the resistance toward 23 tested antibiotics were significantly different (F=6.780, P<0.05) from one another. The mean resistance levels toward penicillin G and ampicillin were significantly higher (P <0.05) than the other tested antibiotics such as chloramphenicol, tetracycline, streptomycin, gentamycin, erythromycin, cephalothin, nitrofurantoin, tobramycin, rifampicin, kanamycin, sulphamethoxazole/trimethoprim, amikacin, imipenem, ceftriaxone, doxycycline, ceftazidime, norflaxacin, vancomycin, piperacillin, ciprofloxacin, and nalidixic acid.

DISCUSSION

Bacterial colony count was performed to determine the total number of microorganisms present in the collected swiftlet feces samples. The $\log_{10}$ of mean bacterial colony count per g was the highest (9.22±0.72) in samples collected from Kota Samarahan (Table 1), mostly because these facultative anaerobic bacteria were capable to grow both aerobically and anaerobically. Besides, Kota Samarahan is situated at the coastal area which has sufficiently nutrient-rich water considering the shallow coastal depth water-level and the abundance of sunlight in this are, thus more conducive for bacterial growth. The present bacterial count was higher than the findings reported by Nyakundi and Mwangi (2011). In their report, the total bacterial viable count was between 2.4 x 10^6 and 7.8 x 10^6 CFU g^{-1} from Marabou stock (*Leptoptilos crumeniferus*) droppings.

The results of this microbiological examination on the bacterial distribution in the samples of swiftlet feces randomly collected at Kota Samarahan, Semarang, Saratok, Betong, Sarikei, Sibu, Sepinang, Maludam, Miri, and Kuching in Sarawak, Malaysia (Fig 1) revealed that most of these bacterial isolates were Gram positive bacteria. *Staphylococcus* sp. was the most prevalent, followed by *Bacillus* sp., *Lysinibacillus* sp. *B4*, *E. harae*, *Sporosarcina* sp., *E. coli*, *Paenibacillus* sp., *P. aeruginosa*, *L. iarius*, and *Dermacoccus* sp.

Staphylococcus sp. and *Bacillus* sp. were highly prevalent bacteria mainly because they are facultative anaerobic bacteria capable of growing both aerobically

Table 2 Resistance to 23 types of antibiotics among bacteria isolated from swiftlet feces collected at Kota Samarahan, Saratok, Betong, Maludam, Miri, Kuching, Semarang, Sepinang, Sarikei, and Sibu in Sarawak, Malaysia

Antibiotic	Resistance rate (%)										
	Kota Samarahan (n=50)	Saratok (n=50)	Betong (n=50)	Maludam (n=50)	Miri (n=50)	Kuching (n=50)	Semarang (n=50)	Sepinang (n=50)	Sarikei (n=50)	Sibu (n=50)	Mean
Penicillin											
Ampicillin	42	62	38	10	28	42	8	20	16	20	28.60±17.13 ab*
Penicillin G	40	66	72	14	46	64	12	20	16	18	36.80±23.87 a
Piperacillin	4	8	0	0	0	0	4	0	0	0	1.60±2.80 c
Cephalosporin											
Ceftriaxone	42	8	0	4	0	2	2	0	6	0	6.40±12.82 c
Ceftazidime	38	16	12	6	0	2	42	18	18	2	15.11±15.50 bc
Cephalothin	44	20	4	4	18	4	16	12	4	0	12.60±13.07 bc
Phenicol tetracycline											
Chloramphenicol	0	8	0	0	18	0	2	0	0	0	2.80±5.90 c
Quinolones											
Norfloxacin	4	16	0	0	0	0	0	0	0	0	3.45±6.82 c
Nalidixic acid	4	8	0	0	0	0	0	0	0	0	1.20±2.70 c
Fluroquinolone											
Ciprofloxacin	4	16	4	0	4	2	0	0	0	0	3.00±4.92 c
Macrolide											
Erythromycin	0	30	0	2	4	10	2	0	8	0	5.60±9.28 c
Aminoglycoside											
Gentamicin	54	20	4	0	0	0	2	20	18	0	11.80±17.22 bc
Kanamycin	2	8	0	0	0	22	0	0	0	0	3.20±7.07 c
Amikacin	0	8	0	0	0	0	8	0	0	0	1.60±3.37 c
Tobramycin	0	8	0	0	0	22	0	0	0	0	3.00±7.13 c
Carbapenem											
Imipenem	0	8	0	0	0	0	0	0	0	0	0.80±2.53 c
Nitrofuran											
Nitrofurantoin	0	16	0	0	18	22	2	0	0	0	5.80±9.02 c
Ansamycin											
Rifampicin	44	28	4	6	24	26	20	9	6	2	16.90±13.70 bc
Streptomycin	4	8	0	0	0	0	10	0	0	0	2.20±3.82 c

* means with the same superscripts are not significantly different at 5% level.

and anaerobically (Clements *et al*. 2002). Besides, *Bacillus* sp. was able to produce endospores that can stay dormant under stressful environment for a long period of time (Wayne *et al*. 2000), thus able to survive even in dry swiftlet feces. Contamination of bird feed in the feces sample could also be the reason for the occurrence of *Staphylococcus* sp. According to Kocijan *et al*. (2009), *Staphylococcus* sp. was ubiquitous and commonly found in bird feed. According to Simpson (2002), it is normal to find *E. coli* in swiftlet feces samples because almost all wild birds have *E. coli* as their normal flora. This study showed that *E. coli* found in the swiftlet feces samples was normal because almost all wild birds contain *E. coli* (Simpson, 2002). Nyakundi and Mwangi (2011) and Literak *et al*. (2007) had isolated *E. coli* from the Marabou Stock (*Leptoptilos crumeniferus*) droppings and rooks droppings samples.

The results, as shown in Table 2, have revealed that most of the bacteria were resistant to penicillin G and ampicillin antibiotics. According to Li and Nikadio (2009), bacteria exhibit an enzymatic deactivation mechanism by producing β-lactamases. In my study, it was shown that *Staphylococcus* sp. was the most highly prevalent bacteria occurring in all the swiftlet houses.

Bozdogan *et al.* (2003) reported that *Staphylococcus* sp. had higher antibiotic resistance tendency toward penicillin, methicillin, tetracycline, and erythromycin. Besides, Cirz *et al.* (2005) showed the similar result showing that *Enterococcus* sp. had higher resistance towards rifampicin. Acquired resistance such as mutation of genes encoding the bacterial protein (Cirz *et al.* 2005; Enne *et al*. 2003) occur and caused antibiotic resistance in most bacteria. Cirz *et al*. (2005) also mentioned that the occurrence of vancomycin resistant *Enterococcus* was high and a lot of researcher had shown the data as early as 1987. *Pseudomonas* sp. was another highly prevalent pathogen and antibiotic resistance bacteria (Poole 2004). *Pseudomonas* sp. exhibits multiple antibiotic resistance mechanisms by developing multidrug efflux pumps encoded by antibiotic resistant genes and bacterial cellular envelopes with lower permeability to antibiotics (Poole 2004). Most of the *E. coli* were highly resistant to piperacillin and streptomycin (Wittwer *et al*. 2005). Streptomycin has been the first-line antimicrobial and widely used as growth promoters in human and agricultural field in Taiwan (Chih *et al*. 2009). Similar result was shown by Chih *et al*. (2009) stated that extensive usage of streptomycin antibiotics caused resistance in *E. coli*.

In conclusion, the mean of bacteria colony count ($\log_{10}$ CFU g^{-1}) was the highest in Kota Samarahan and the lowest in Betong. There were more Gram positive than Gram negative bacteria isolated from the swiftlet feces in Sarawak, Malaysia. *Staphylococcus* sp. and *Bacillus* sp. were the most highly prevalent bacteria in the swiftlet farm houses. The isolated bacteria showed high resistance to penicillin G and ampicillin when tested against 23 types of antibiotics commonly used in current medical practice. If the antibiotic resistant bacteria cannot be controlled or killed by antibiotics, it might become a serious threat to public health.

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