UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF STREPTOCOCCUS EQUI EQUI IN GUTTURAL POUCH IN HORSES

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ISOLATION AND IDENTIFICATION OF STREPTOCOCCUS EQUI EQUI IN GUTTURAL POUCH IN HORSES

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A project paper submitted to the

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "Isolation and Identification of *Streptococcus equi equi* in Guttural Pouch in Horses", by Norwahidah binti Alias and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999 –Final Year Project.

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DEDICATION

I wish to dedicate this Final Year Project to my mother Kamariah bt Mohd,

my father, Alias bin Ali

my sisters, Noraslinda and Norafizah

Thank you for your endless support throughout my project.

Also to Dr. Zainita Mohd Noor

for the continuous words of motivation, guidance and attention.

My best friends, Siti Hawa, Farasiha and Hidayah

for their love and care

May this be your inspiration and motivation for your future endeavours

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ABSTRACT

An abstract of the project paper presented to the Faculty of the Veterinary Medicine in partial fulfillment of the course VPD 4999 –Project.

ISOLATION AND IDENTIFICATION OF STREPTOCOCCUS EQUI EQUI IN GUTTURAL POUCH IN HORSES

by

Norwahidah bt Alias

2015

Supervisor: Assoc. Prof. Dr. Md Sabri Mohd Yusoff

Co- supervisor: Dr. Nurul Hayah Khairuddin

Guttural pouch is a paired extension of auditory tube which is known to be one of the structures of upper respiratory tract in horses. Any changes in the microflora along the respiratory tract can lead to respiratory disease under certain circumstances. There is limited information on bacteria isolates in guttural pouch of horses from tropical countries and this study was conducted to identify *Streptococcus equi equi* and other normal flora in the guttural pouch of horses. A total of 4 euthanized horses were used in this study and surgical approach of Viborg's triangle technique was performed to get access to the

guttural pouch. A sterile PBS was instilled into the guttural pouch to obtain the samples. The samples were cultured onto blood agar and McConkey agar and identified by a series of biochemical test. Seventeen isolates obtained which comprised of 9 species of bacteria and they were identified as *Staphylococcus aureus* (17.65%), *Klebsiella pneumonia* (17.65%), *Stapyhlococcus intermedius* (11.76%), *Pasturella spp.* (11.76%), *Corynebacterium spp.* (11.76%), *Actinomyces spp*. (11.76%) and *Pasturella caballi* (5.88%), *Moraxella equi* (5.88%), and *Rhodococcus spp.* (5.88%). However, *Streptococcus equi equi* was not among the isolates obtained. The Polymerase Chain Reaction (PCR) employed confirmed, negativity for *Streptococus equi equi*. Based on the findings of this study, it was concluded that several bacterial flora reside in the guttural pouch in apparently healthy horses and majority of bacteria are similar with those in upper respiratory tract reported in temperate countries which could become pathogenic in immunocompromised or stressed horses.

Keywords: Horses, immunocompromised, lavage, respiratory tract, Viborg's triangle.

ABSTRAK

PENGASINGAN DAN PENGENALPASTIAN STREPTOCOCCUS EQUI EQUI DI DALAM KANTUNG GARAU KUDA

oleh

Norwahidah bt Alias

2015

Supervisor: Assoc. Prof. Dr. Md Sabri Mohd Yusoff

Co- supervisor: Dr. Nurul Hayah Khairuddin

Kantung garau dikenali sebagai sepasang tiub auditori lanjutan dan merupakan salah satu struktur di dalam sistem pernafasan di kuda. Setiap perubahan pada mikro flora di sepanjang saluran pernafasan kuda dapat menyebabkan terjadinya masalah pada sistem pernafasan pada kuda dalam keadaan tertentu. Maklumat mengenai bakteria di dalam kantung garau adalah sangat kurang dilaporkan oleh negara-negara tropika. Kajian ini dijalankan untuk mengenalpasti kehadiran bakteria *Streptococcus equi equi* dan bakteria lain di dalam kantung garau. Sejumlah 4 ekor kuda eutanasia digunakan di dalam kajian ini. Teknik yang digunakan adalah Segi tiga Viborg untuk mendapatkan kantung garau. Larutan phosphate buffer dimasukkan ke dalam kantung garau untung mendapatkan

sampel air daripada kantung garau. Sampel tersebut dikultur pada agar darah dan agar McConkey dan identiti bakeria dikenalpasti melalui ujian biokimia. Sebanyak 17 bakteria dijumpai yang terdiri daripada 9 jenis bakteria iaitu *Staphylococcus aureus* (17.65%), *Klebsiella pneumonia* (17.65%), *Stapyhlococcus intermedius* (11.76%), *Pasturella spp*. (11.76%), *Corynebacterium spp*. (11.76%), *Actinomyces spp*. (11.76%) and *Pasturella caballi* (5.88%), *Moraxella equi* (5.88%), and *Rhodococcus spp*. (5.88%). Walau bagaimanapun, tiada *Streptococcus equi equi* ditemui daripada sampel kantung garau. Ini dipastikan lagi dengan keputusan negatif pada kaedah PCR (Reaksi Rantai Polimer). Terdapat pelbagai normal bakteria yang mendiami kantung garau kuda yang sihat dan kebanyakan daripadanya adalah bakteria yang sama dengan bakteria pada saluran pernafasan kuda yang banyak dilaporkan oleh negara –negara beriklim sederhana. Bakteria –bakteria ini akan menyerang sekiranya haiwan mengalami tekanan dan imunisasinya terganggu.

Kata kunci : kuda, saluran pernafasan, imunisasi, Segi tiga Viborgs, *lavage*

1.0 INTRODUCTION

Guttural pouch is one of the structure of upper respiratory tract in horses as well as others such as nasal passages, nasopharynx, larynx and trachea. Horses are obligate nasal breathers due to their anatomical structure of complete separation of nasopharynx and oropharynx. Thus, it is important to keep the airways structures in good condition and function as normal to maintain the health of the horse and for them to achieve their best performance and athletic activity.

Respiratory tract has abundance of normal flora such as aerobes, anaerobes bacteria and fungus. The normal microflora will flare up under some circumstances and compromised the local immunity of the airway structure and lead to disease. However, *Streptococcus equi* subspecies *equi* which is known to cause strangles (Hardy *et al*., 2005) is not one of normal flora of the airway tract in horses. It is from purulent discharges of infected horse or fomites which enters via the mouth or nostrils of susceptible horses (Sweeney *et al*., 2005; Waller, 2014). Then, the bacteria will attach to the cells in the crypt of the tonsils and adjacent lymphoid nodules. (Taylor and Wilson, 2006). Besides, the pharyngeal opening of the guttural pouches are open during swallowing and dilatation of the openings can exposed the pouches to many pathogenic organisms and will lead to infection. (Edwards and Greet, 2007).

Strangles is an infectious respiratory disease of horses and a worldwide problem due to its causal agent which is difficult to eliminate and create persistence infection. The

source of infection can be from the apparently healthy horse who carry the organism in the guttural pouch without showing any clinical sign, horses that recently recover from the disease and apparently sick horses. (Sweeney *et al*., 2005). Abscessation of retropharyngeal lymph nodes due to accumulation of extracellular organisms, degenerating neutrophils and necrotic tissue will then rupture into guttural pouch causing empyema. (Taylor and Wilson, 2006). Then purulent materials will drain into nostrils, giving the clinical signs of nasal discharges associated with strangles. Enlarged lymph nodes will compress the pharynx, larynx and trachea and results in the obstruction of the respiratory tract.

There are numerous literatures available on existence of the *Streptococcus equi* subspecies *equi* in guttural pouch of horses in temperate countries (Taylor *et al*., 2006; Webb *et al*., 2012; Parillo *et al*., 2007; Sweeney *et al*., 2005). However, there is lack of literature on *Streptococcus equi* subspecies *equi* and normal bacterial flora in guttural pouch of horses from the tropical countries. Therefore, this study is beneficial to detect asymptomatic carrier to provide future control and management of the disease. Other than that, it is important to determine bacterial flora in the airway tract as they will flare up and cause disease in immune compromised horses.

The objectives of this project were:

1. To isolate and identify *Streptococcus equi* subspecies *equi* in guttural pouch of horses.
2. To isolate and identify the bacterial flora population in the guttural pouch of horses.
3. To study the histological structure of the guttural pouch in horses.

Hypothesis of this study were:

1. Streptococcus equi equi are presence in guttural pouch in horses.
2. Bacteria flora in the guttural pouch are similar to those bacterial flora from the respiratory tract.

2.0 LITERATURE REVIEW

2.1. Guttural pouch

The guttural pouch is a paired diverticula of the Eustachian tube of left and right which connect pharynx to the middle ear. Each is separated into larger medial and smaller lateral compartment by stylohyoid bone and has the capacity of 300-500 ml. (Rush and Mair, 2004). Medial compartment is in contact with internal carotid artery, cranial cervical ganglion and cranial nerves such as glossopharyngeal nerves (IX), vagus nerve (X), accessory nerve (XI) and hypoglossal nerves (XII) while the lateral compartment is in contact with the external carotid artery. On the ventral and lateral aspect of medial compartment, there is medial retropharyngeal lymph node and lateral retropharyngeal lymph nodes respectively which it is lies beneath the mucosa. (Freeman *et al*., 2011; Edward *et al*., 2009; Hardy *et al*., 2005). If there is abscessation of retropharyngeal lymph nodes especially due to *Streptococcus equi equi*, it will then rupture into guttural pouch causing empyema. *Streptococcus equi equi* can be detected in the guttural pouch during outbreak and the organism reside in the guttural pouches of the carrier horses(Newton, 2000).

The guttural pouch is covered laterally by the pterygoid muscle, parotid gland and mandibular glands. (Dyce *et al*., 2010). The left and right guttural pouch are opposed forming a thin median septum. However, at the dorsal parts of the pouches, they are separated by the rectus capitis and longus capitis muscles. (Dyce *et al*., 2010; Hardy and Levielle, 2003).

The pharyngeal openings of the pouches which appear as slit -like openings lie on the dorsolateral wall of the pharynx and rostroventral to the pharyngeal recess. It is funnel shape opening into the guttural pouch. Besides, the openings are located at dorsal aspect of the pouches thus, the horse' head need to be lowered to obtain drainage besides, it is open when the horse is swallowing. (Edwards and Greet, 2007)

The exact function of the guttural pouch is still uncertain. However, it has been proposed that it serves as brain cooling to dissipate heat as there is ventilation of the pouch that will results in blood cooling of internal carotid artery (Baptiste, 1998). It is also believed that the function is to equalize air pressure on both sides of the tympanic membrane (Rush & Mair, 2004). Besides, there is exchange of air in the guttural pouch during respiration and the pharyngeal opening is dilate during swallowing. Other than that, it is a resonating chamber for vocalization and a floatation device. (Briggs, 2000).

2.2. Normal bacterial flora in the guttural pouch

There are a lot of literatures on normal bacterial flora in the upper respiratory tract reported in temperate countries which inhabit the upper respiratory tract of apparently healthy horses such as *Bacillus spp.*, *Streptococcus zooepidemicus, Staphylococcus aureus, Staphylococcus intermedius, Corynebacterium spp., Micrococcus spp., Rhodococcus equi, Pseudomonas spp., Klebsiella pneumonia, Pasturella spp., Bordetella spp.*, and *Escherichia coli* (Mir *et al.*, 2013; Debelu *et al.*, 2014). The inhabitants in

respiratory tract in horses are *Streptococcus zooepidemicus, Pasturella spp., Escherichia coli,* and *Actinomyces spp.* (Hardy and Davis, 2014). However, there is limited literature on bacterial flora in the guttural pouch in tropical and temperate countries. Majority of the bacteria found from a cytological and bacteriological analysis from guttural pouch of ten slaughtered horses were similar to those bacteria reported from respiratory tract. Besides, *Moraxella* sp was also isolated from the guttural pouch (*Chiesa et al.,* 1999). According to Higgins, the similar isolation of bacteria in guttural pouches and respiratory tract is not unexpected considering the continuity between the pharyngeal and guttural pouches mucosa (Higgins *et al.,* 2000)

2.3.*Streptococcus equi* subspecies *equi* organism

Streptococcus equi subspecies *equi,* the causative agent of Strangles, is a gram positive cocci bacterium that appears in chains or pairs. Besides, it forms honey -coloured mucoid colonies on a blood agar with a wide zone of beta hemolytic properties. However, its colony morphology can be similar to *Streptococcus zooepidemicus*. In addition, both of them are Lancefield Group C. Therefore, *Streptococcus equi* is often differentiated from *Streptococcus zooepidemicus* by its inability to ferments lactose and sorbitol in the biochemical test. Despite of that, some strains of Streptococcus zooepidemicus also unable to ferment these sugars. Thus, DNA -based test as Polymerase Chain Reaction (PCR) is more sensitive to differentiate between these organisms. (Hines *et al.,* 2014).

Streptococcus equi subspecies *equi* has SeM protein in the capsule which capable of inhibiting complement deposition and prevents opsonization. Furthermore, SeM protein and hyaluronic acid capsule produced by the bacterium has anti- phagocytic properties that prevents phagocytosis by the macrophage and neutrophils (Taylor *et al*., 2006; Sweeney *et al*., 2005).

As the bacteria entering the host by inhalation or ingestion from the mucopurulent discharge of infected horses or fomites, the bacteria will attaches to the cells at crypts of the tonsils and adjacent lymphoid nodules. The bacteria produce enzymes and toxins then causing damage to the surrounding cells and activate inflammation. Due to ability of the bacterium to avoid immune response, it will lead to the accumulation of extracellular bacteria in the form of long chain with a large numbers of degenerating neutrophils and causing abscess. (Boyle, 2011; Sweeney et al., 2005)

2.4. Histological structure of guttural pouch mucosa

There is lack of information on the histological structure of guttural pouch mucosa reported in tropical countries. However, there are several literatures available from temperate countries on the histology of the guttural pouch. The guttural pouch is lined with pseudostratified ciliated epithelium containing goblet cells. The structures were presence in both adults and foals although there were some underdeveloped area in the foals. The guttural pouch mucosa capable of clearing the foreign substances depending on different regions of the epithelium (Freeman and Hardy, 2012). Other than that, goblet cells were scattered throughout the guttural pouch regions and they were abundant especially near the pharyngeal opening of the guttural pouch. In the postero –superior and postero –medial walls, there was thick epithelial layer and thin lamina propria. It suggested that, the thin lamina propria consisted lesser development of glands. (Manglai *et al*., 2000)

3.0 MATERIALS AND METHODS

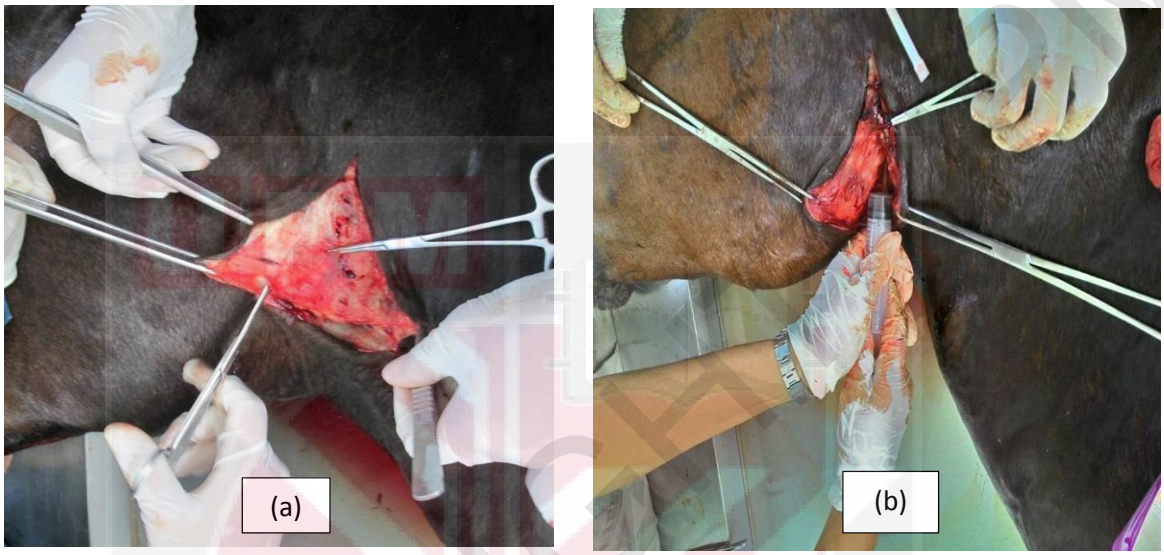
3.1 Sampling of guttural pouch lavage

A total of 4 euthanized horses were used in this study. The horses were euthanized using Dolethal 200mg/ml and placed on lateral recumbency. Endoscope was introduced into a nostril and extending up the ventral nasal meatus to the level of common pharynx. The pharyngeal openings of the guttural pouch are visible as mucosal flaps on dorsolateral of the pharynx and endoscope was slightly twisted to help entry to the pouch. The mucosa of the pouch was visualized and examined for any pathological lesion.

Surgical approach to obtain guttural pouch lavage was performed using Viborg's triangle technique. Viborg's triangle is made up by tendon of sternocephalicus muscles, vertical ramus of mandible and the linguofacial vein. A vertical incision is made in the triangle area (Freeman & Hardy, 2012) as shown in **Figure 1 (a)**. Then, a blunt dissection is made through the parotid gland, to get through the wall of the guttural pouch. Once the compartment wall of the guttural pouch is determined, 18G needle and 20 ml syringe was used to instill about 100ml of PBS into the pouch and the lavage was aspirated out. The guttural pouch lavage was placed into a sterile containers. The same procedure was repeated for the other side of guttural pouch.

The samples were brought to Veterinary Bacteriology Laboratory in Faculty of Veterinary Medicine on the same day. All the samples were centrifuged in 4000 rpm in 10 minutes. The supernatant was discarded leaving the sediments.

Figure 1: (a) Vertical incision in the Viborg's triangle. (b) The withdrawal of the guttural pouch lavage



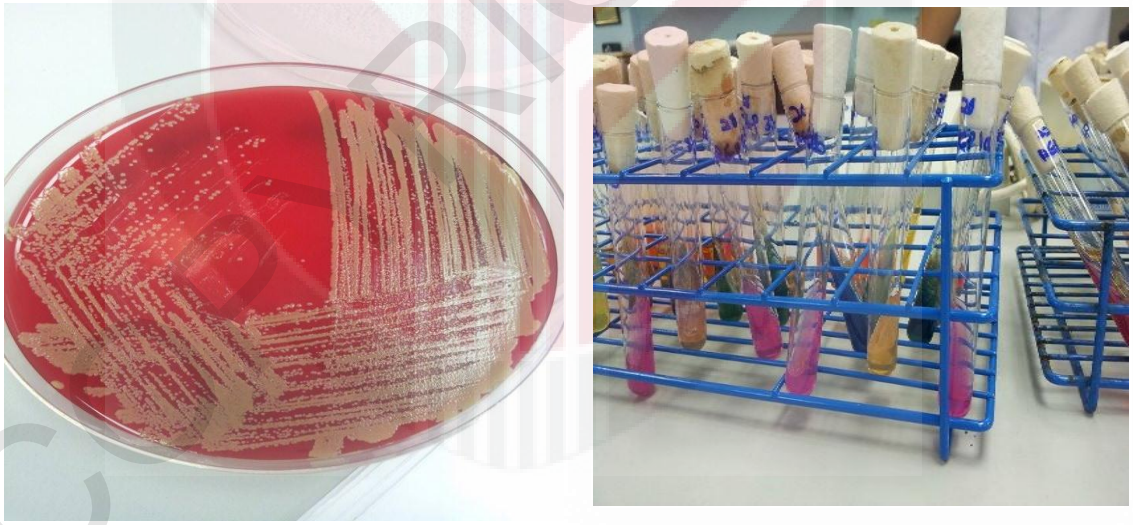
3.2 Sampling of guttural pouch tissue

The horse's head was severed at the level between atlas and axis to the neck region preserving the guttural pouch area. The guttural pouch is located between the base of the cranium dorsally and the pharynx and esophagus ventrally (Rush and Mair, 2004). The tongue and larynx was severed and separated out from the buccal cavity to expose the wall of the pharynx. On the dorsolateral wall of the pharynx, there was a slit-like opening of the guttural pouch on both sides located rostroventral to the pharyngeal recess. Endoscope was introduced into the pharyngeal opening to visualize and examine the mucosa of guttural pouch. Several tissues of guttural pouch wall were taken and placed into a bottle containing 10% formaldehyde as fixative for the histological studies.

3.3 Isolation procedure

A loopful of lavage sediment from the centrifuge tube was streak onto Blood Agar and McConkey Agar and incubated for 24 -48 hours at 37°C. All the colonies isolated in the primary culture were then cultured onto Blood agar and McConkey for subculture. Each colonies isolated were Gram stained and the cell morphology such as shape, color and distribution of the bacteria were recorded. Then, all the colonies were subjected to a series of biochemical tests for identification.

Figure 2: (a) Pure culture of *Staphylococcus* spp. (b) Biochemical test results after 24 hours incubation



3.4 Identification of bacteria

All the pure colonies were then grouped based on their Gram stained which was gram positive cocci, gram positive rods or gram negative bacteria and subjected to their respective biochemical test. The biochemical test for gram positive cocci bacteria, gram positive rods and gram negative bacteria were shown in **Table 1, Table 2** and **Table 3** respectively.

Table 1: Biochemical tests for Gram positive cocci bacteria

Biochemical test	Method
1. Catalase test	A loop of bacteria was mixed with a drop of hydrogen peroxide on a glass slide. Presence of bubbles indicates positive test
2. Coagulase test	A drop of normal saline was mixed with a loop of bacteria on a glass slide. Then, a drop of rabbit plasma was placed on the bacteria suspension. Presence of clumps within 30 seconds indicate positive test
3. Blood broth	A loop of bacteria that give coagulase test positive or catalase test negative was inoculated into the blood broth tube and incubated for 24 hours at 37°C. Degree of hemolysis was observed. Red to brownish reaction indicate positive test.

4.	**VP**	A loop of bacteria that give coagulase test positive was inoculated into VP tube and incubated for 24 hours at 37°C. After incubated, 6 drops of alpha -naphtol was added together with 4 drops of 40% KOH and shake the tube. Red color indicates positive test.
5.	**Maltose and Mannitol**	A loop of bacteria that give coagulase test positive was inoculated into Maltose and Mannitol tube and incubated for 24 hours at 37°C. Yellow color indicates positive test.
6.	**ADH**	A loop of bacteria that give coagulase test positive was inoculate into ADH tube. A layer of mineral oil was added into the tube. The tube was incubated for 24 hours at 37°C. Purple color indicates positive test.
7.	**6.5% NaCl**	A loop of bacteria that give catalase test negative was inoculate into 6.5% NaCl tube and incubated for 24 hours at 37°C. Any changes in color from the original purple color indicates positive test
8.	**Bile esculin**	A loop of bacteria that give catalase test negative was streaked onto slant bile esculin agar. The tube was incubated for 24 hours at 37°C. Black color on the streaking indicates positive test.

9.	**Lactose, Sorbitol, Trehalose**	A loop of bacteria that give catalase test negative was inoculated into Lactose, Sorbitol and Trehalose tubes and incubated for 24 hours at 37°C. Yellow color indicates positive test.

Table 2: Biochemical tests for gram positive rods bacteria

Biochemical test		Method
1.	**Catalase test**	A loop of bacteria was mixed with a drop of hydrogen peroxide on a glass slide. Presence of bubbles indicates positive test
2.	**Blood broth**	A loop of bacteria that give coagulase test positive or catalase test negative was inoculated into the blood broth tube and incubated. Degree of hemolysis was observed. Red to brownish reaction indicate positive test.
3.	**Glucose, Sucrose, Trehalose**	A loop of bacteria that give catalase test positive was inoculated into glucose, sucrose and trehalose tubes. The tubes were incubated for 24 hours at 37°C. Yellow color indicates positive test.

4.	**Urease**	A loop of bacteria that give catalase test positive was streaked onto urea agar.Pink color indicates positive test.
5.	**Nitrate**	A loop of bacteria that give catalase test positive was inoculated into nitrate tubes and incubated 24 hours at 37˚C. After incubated, 3 drops of Nitrate 1 and Nitrate 2 were added. Red color indicates positive test.
6.	**SIM**	A loop of bacteria that give catalase test positive was inoculated into the tubes and incubated. After incubated, 4 drops of Kovacs reagent was added. Red color indicates positive test for indole. Cloudiness of the agar indicates positive test for motility. Black color of the agar indicates positive test for sulphide.

Table 3: Biochemical tests for Gram negative bacteria

Biochemical test	Method
1. **Oxidase test**	Oxidase reagent was placed on the filter paper and mixed with a loop of bacteria. Purple color indicates positive test
2. **TSI**	A loop of bacteria was streaked and inoculate into agar and incubated. Yellow color indicates acid reaction, purple to red

		color indicates alkali reaction and black color indicates presence of H2S. Noted also the presence of gas
3.	**SIM**	A loop of bacteria was inoculated into agar and incubated. Cloudiness indicates positive test for motility. Black color indicates positive sulphide. Four drops of Kovacs reagent was added. Red color indicates positive indole.
4.	**Citrate**	A loop of bacteria was streaked onto agar and incubated. Blue color indicates positive test.
5.	**Urea**	A loop of bacteria was streaked onto agar and incubated. Pink to purple color indicates positive test.

3.5 Polymerase Chain Reaction (PCR)

A representative of colony from each pure culture was inoculated in Brain Heart Infusion broth and incubated for 24 hours at 37°C. Then, DNA extraction was performed according to protocol by Wizard Genomic DNA Purification Kit. Mastermix for PCR was prepared before added into the sample containing DNA of bacteria for each 4 horses. The PCR product was placed into mini cycler machine to amplify the DNA fragment for *Streptococcus equi equi* with primer sequence as shown in **Table 4**. (Newton *et al*., 2000)

The cycling condition for PCR -1 and PCR -2 described as follows: 1 cycle at 95 °C for 10 minutes, 30 cycles of 92 °C for 1 minute, 58 °C for 1 minute,72 °C for 1 minute and followed by a period of 5 minutes at 72 °C. The thermocycling condition for PCR -

3 described as: first cycle at 95°C for 10 minutes followed by 30 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1minute 30 seconds followed by a period of 5 min at 72°C. In a meanwhile, 2% agarose gel was prepared and ready to be used after 20 minutes. Loading dye buffer was mixed with PCR product and placed into its respective well for electrophoresis at 115 V in 45 minutes. Gel was examined on a UV transilluminator.

Table 4: Primers used in PCR test of *Streptococcus equi equi*

Test	Round of each reaction	Primer sequence (5'-3')
PCR -1	First	TGCATAAAGAAGTTCCTGTC;
		GATTCGGTAAGAGCTTGACG
	Second	CATACCTATCTCCATCAGCA;
		CGAACTCTGAGGTTAGTCGT
PCR -2	First	GCATAAAGAAGTTCCTGTCATTAAAAT;
		CGGTAAGAGCTTGACGCTCATCTT
	Second	ATACCTATCTCCATCAGCAATCCG;
		CTCTGAGGTTAGTCGTACGGCGA

3.6 Histology of guttural pouch

Tissues of the wall of the guttural pouch was placed into the cassettes after sliced into small portion. It was processed into an alcohol series overnight before embedded in paraffin. Then, the tissue was sectioned at 4-5µM using microtome and stained with hematoxylin and eosin (H&E) in **Figure 3.** The stained slides were covered with thin cover slip to protect the tissues beneath. The slide was examined under light microscope.

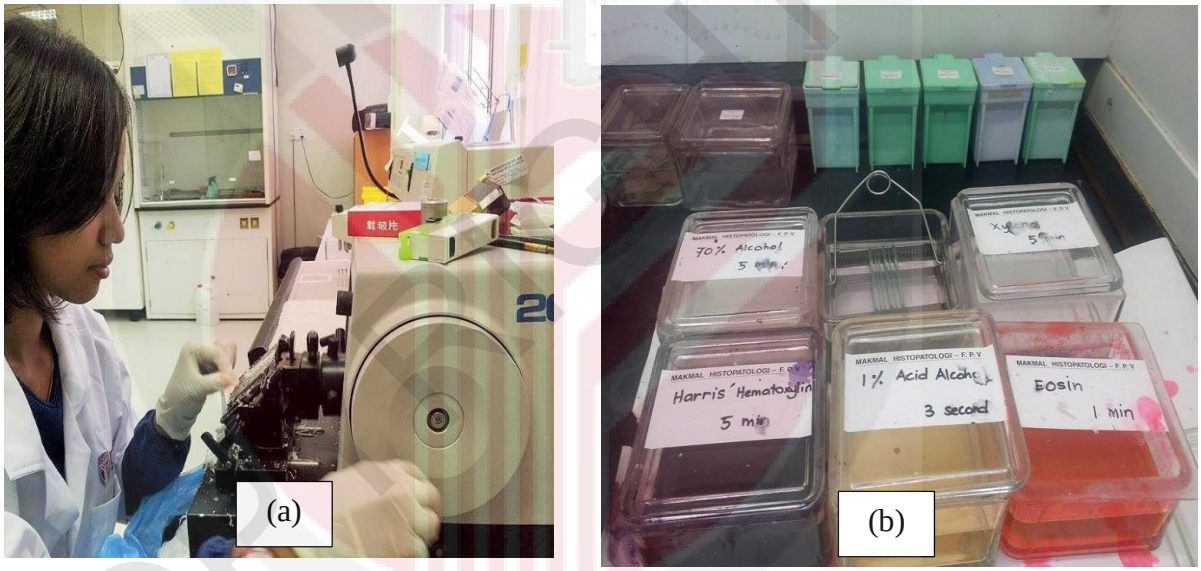


Figure 3 (a): Sectioning of tissue using microtome (b) H & E staining

4.0 RESULTS

4.1 Bacteria isolation and identification

Seventeen isolates were obtained which comprised of 9 species of bacteria. The bacteria identified in guttural pouch can be shown in **Table 4**

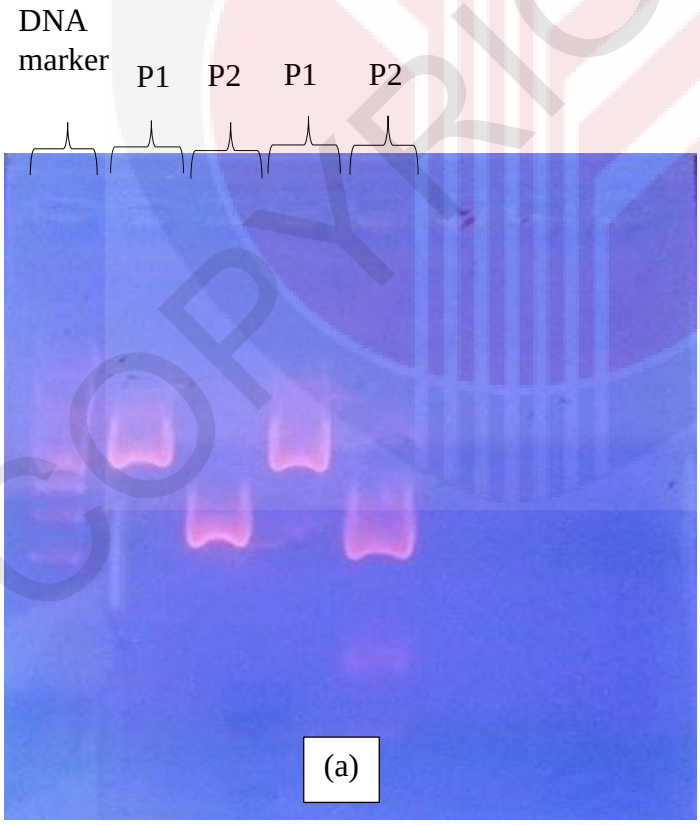
Table 4: Bacteria isolates in guttural pouch in horses

Bacteria	**No. of samples with isolates**	**Percentage (%)**
Staphylococcus intermedius	2	11.76
Staphylococcus aureus	3	17.65
Moraxella equi	1	5.88
Rhodococcus spp.	1	5.88
Pasturella spp.	2	11.76
Pasturella caballi	1	5.88
Actinomyces spp.	2	11.76
Klebsiella pneumonia	3	17.65
Corynebacterium spp.	2	11.76
Total	**17**	**100**

4.2 PCR

Polymerase Chain Reaction (PCR) tested all DNA samples from four horses for *Streptococcus equi equi*. A positive control from the pure culture of *Streptococcus equi equi* was tested and showed PCR detecting M-gene (SeM) can be conducted. Primers that are specific for *Streptococcus equi equi* were used to test sample from 4 horses and they were all negative for *Streptococcus equi equi.*

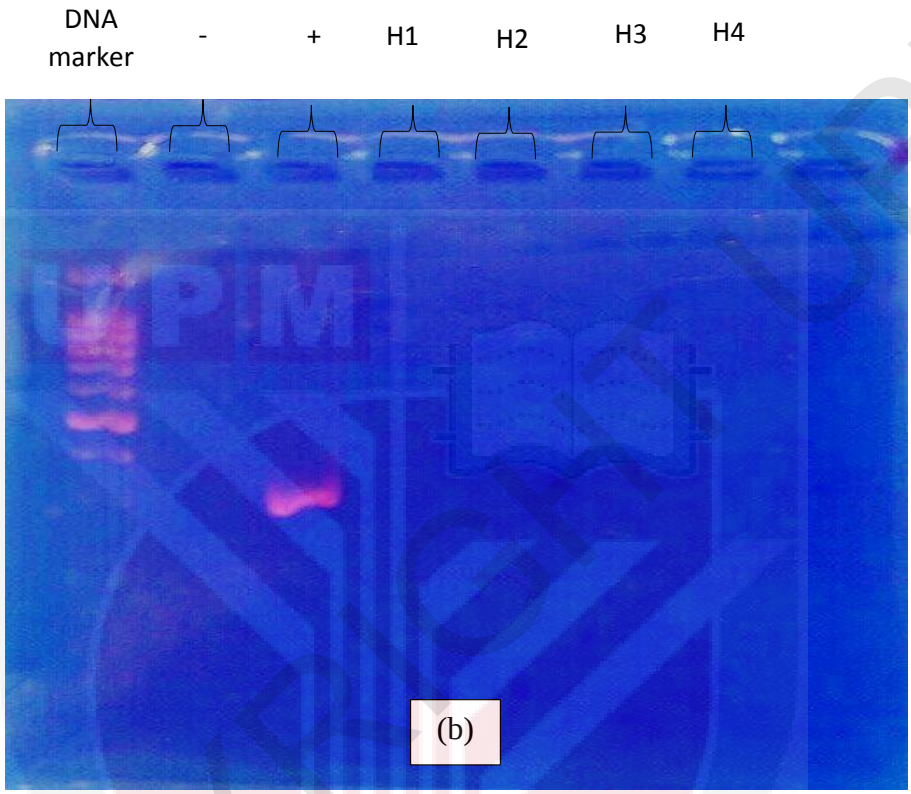
Figure 4: (a) Positive control using Primer 1 and Primer 2 (b) Negative results of the sample shown using Primer 2



DNA marker 100bp

P1 : Primer 1

P2 : Primer 2



DNA marker 100bp

- : negative control

+ : positive control

H1 : DNA sample from Horse 1

H2 : DNA sample from Horse 2

H3 : DNA sample from Horse 3

H4 : DNA sample from Horse 4

4.3 Histology of the wall of guttural pouch

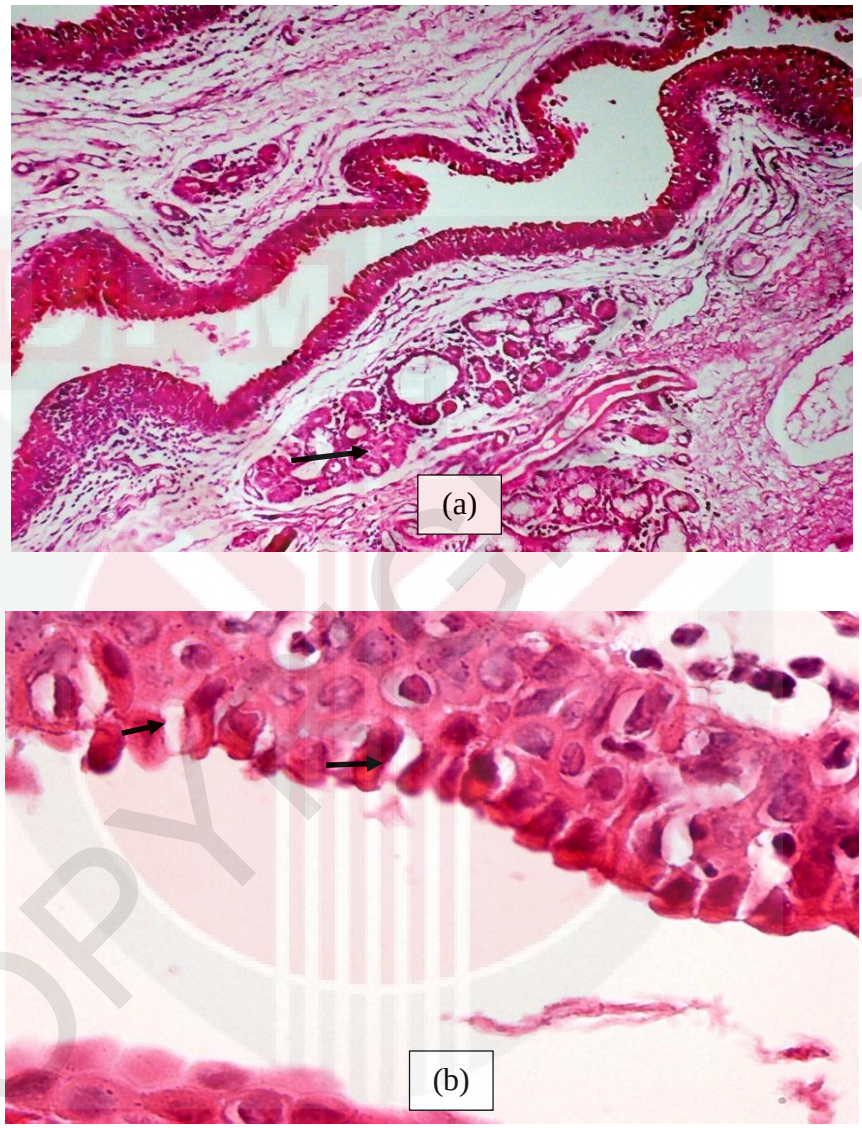


Figure 5 (a) Samples taken from medial compartment of guttural pouch. Presence of glands and vessels in the lamina propria. (H&E, x20) (b) Guttural pouch is lined by pseudostratified columnar epithelium containing goblet cells. (H&E, x40)

5.0 DISCUSSION

Majority of the isolates obtained were in agreement to those bacteria from the respiratory tract reported in temperate countries. However, 2 out of 17 isolates obtained were unusual and less commonly reported. They were *Moraxella equi* and *Pasturella caballi. Moraxella equi* was isolated from the eye of horse and also in horses with conjunctivitis (Dworkin *et al*., 2006). Besides, *Pasturella caballi* is the commensals organism of upper respiratory tract mucosa and it was isolated in high number from horses with upper respiratory tract infection (Jeans *et al.,* 2006). The highest isolates obtained which were *Staphylococcus aureus* and *Klebsiella pneumonia. Staphylococcus aureus* is a member of commensal microflora in many animals (Burton *et al.,* 2008) while *Klebsiella pneumonia* can be found in soil and water and it was isolated higher in severe respiratory cases (Eguchi, 1989). *Corynebacterium spp*., *Actinomyces spp*., *Staphylococcus intermedius* each isolated 11.76% were the bacterial flora of the upper respiratory tract of horses (Davis *et al.,* 2014). *Corynebacterium spp.* is pyogenic bacteria located on mucus membranes and skin of animals and known to cause a suppurative condition in equine. (Quinn *et al.,* 2002; Sweeney *et al.,* 1991). *Staphylococcus intermedius* was isolated higher in apparently healthy horses and lower in horses with respiratory problems (Mir *et al.,* 2013). Other than that, *Rhodococcus spp* was among the isolates obtained and it is known to be an opportunistic pathogen and common soil inhabitant. Besides, it is common isolates of respiratory tract (Quinn *et al.,* 2002). Among all the isolates obtained, it revealed that gram positive bacteria were more than gram negative bacteria. However, there is lack of literatures and reasons regarding the results.

Perhaps in the respiratory tract, there are wide distribution of glycosaminoglycans which can be ligands and determinant for the adhesion of the gram positive bacteria especially *Streptococcus equi.* (Gutierrez, 2013). From the findings, it should benefits the clinician to consider the dominance of gram positive bacteria in the upper respiratory tract primarily as a cause of upper respiratory disease in the selection of antibiotics when treating horses with respiratory problem (Debelu et *al.*, 2014).

The use of PCR in this study is to serve as a confirmatory test which increases the carrier detection rate. The test is three times more sensitive than cultures. However, it cannot correlate with an active infection. A positive test can be DNA from live or death bacteria. (Gutierrez, 2013). Pure colony of *Streptococcus equi equi* on Blood agar which act as positive control was tested and showed PCR detecting M-gene (SeM) can be conducted. However, none of the samples were positive for *Streptococcus equi equi.*

Histologically, the guttural pouch wall is lined by pseudostratified ciliated epithelium containing goblet cells. However, presence of cilia cannot be appreciated from the histology slide with H & E staining. Perhaps the use electron microscopy (EM) was the most preferable method in the study.

6.0 CONCLUSION

From this descriptive study, there were 17 isolates obtained which comprised of 9 species of bacteria and the objective stated was achieved. However, *Streptococcus equi equi* was not among the isolates obtained. Thus, the hypothesis was not accepted. Bacteria culture and identification is a gold standard in detecting presence of *Streptococcus equi equi*. However, Polymerase chain reaction (PCR) can serves as confirmatory test which has better sensitivity. Thus, PCR can be carried out in parallel to bacterial culture to increase detection rate of carriers.

7.0 RECOMMENDATIONS

It is recommended that a bigger sample size to be used in the study to isolate *Streptococcus equi equi*. Besides, it is to represents the population and bigger sample size provides more reliable data on the bacterial flora in the guttural pouch. Other than that, include the samples from live animals and samples are taken from different places. To enhance *Streptococcus equi equi* growth, 5% horse blood agar supplemented with colistin sulphate and oxolinic acid can be used (Waller, 2014). Furthermore, the guttural pouch lavage samples are directly subjected to PCR test and positive PCR samples are confirmed with bacterial culture of the guttural pouch lavage to isolate *Streptococcus equi* (Boyle, 2011). As for histology, electron microscopy is the most preferable in examine the mucosa of the wall of guttural pouch.

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