Isolation and Identification of *Trichophyton verrucosum* from Horses in Michael Okpara University Farm, Umudike, Abia State, Nigeria

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Abstract: Fungi like *Trichophyton* species have been implicated in economic loss in the livestock industry. Spread of this organism from one animal to the other and its zonotic nature necessitates this study. The aim of the study was to isolate and identify *Trichophyton verrucosum* from horses. The study was conducted in the Michael Okpara university farm. Three different sample types from 4 horses were used for the study and these include fecal, blood and skin scrappings. Floatation technique, hematological analysis and serum biochemistry test and Needle mount test were used. Phenotypically, the horses were emaciated with prominent rib cage at the left and right flank of the thoracic vertebrae, areas of alopecia on the left flank of the ventral lumbar vertebrae of the hind limb and ball and socket joint of the femur. The heart rate was high at an average of 67beats/min. There were no endoparasites (eggs, larvae and adult worms) detected in the feaces, low pack cell volume (28%), absence of monocytes, high aminotransferase (AST) at 612µ/L, high creatinine and blood urea nitrogen (BUN). Ectoparasites (ticks and mites) were absent. Biochemically, T. *verrucosum* hydrolyses urea. Macroscopically, the texture of the growth organism was waxy and cottony with white coloration. Microscopically, septate, conidospore, macroconidia, microconidia were visible and the arrangement of the chlamydospores was remarkable and used for identification of T. *verrucosum*.
Keywords: T. *verrucosum*, equine, endoparasites, ectoparasites, hemanalysis, and serum biochemistry.

INTRODUCTION

Equine fungal diseases are classified into four different forms/types; namely superficial mycosis, caused by microorganisms limited to the stratum corneum, cutaneous mycosis, caused by pathogens restricted to the keratinized tissues, subcutaneous mycosis and deep mycosis, which affect the respiratory tract and internal organs (De Hong *et al.*, 2002). Commensal pathogens like *Malassezis pachydermatis* had been implicated as a common organism of superficial mycosis in horses (White *et al.*, 2006; Kim *et al.*, 2011). *Trichophyton verrucosum* is a dermatophyte largely responsible for fungal skin disease in cattle, goats, sheep and horses (Beneke and Rogers, 1996). Distribution is worldwide but infection is by contact with infected animals and this could lead to severe inflammation of the affected area (Havlickova *et al.,* 2008; Tyring *et al.*, 2005).

Macroscopically, the growth rate of *Trichophyton* colonies is slow to moderate. The texture is waxy and glabrous to cottony. From the front, the color is white to bright yellow or red (Sutton *et al.*, 1998; Larone, 1995). Microscopically, septate, hyaline hyphae, conidiosphores, microconidia, macroconidia, arthroconidia were observed (St-Germain and Summerbell, 1996). Chlamydospores may also be produced. Macroconidia are often the predominant type of conidia produced by T. *verrucosum* (De Hong *et al*., 2002). T.*verrucosum* transmission occurs by direct contact with clinically affected animals or indirectly from contaminated formites (Roberson and Pugh, 2012).

Dermatophyte which causes dermatophytosis is considered zoonotic because they can be transmitted from animals to humans.

T. verrucosum organism is a filamentous fungus causing mild to severe lesions in horses and sometimes "ringworm" particularly in young horses (Chermette *et al.,* 2008). Dermatophyte infection is acquired by direct contact with diseased animals, asymptomatic carriers, or from the environment.

The clinical signs associated with dermatophyte include alopecia associated with erthyema. Lesions due to *T. verrucosum* are typically dry with thin powdery scales and broken hairs at the base. Miliary dermatitis emerges, extending from the saddle to the girth, then throughout the body in a generalized form (Chermette *et al.,* 2008).

Observation: the horses were always grazing in the field of the university premise, during the raining season. Occasionally, concentrates were fed to the horses, they were observed to have gradually started emaciating, with the presence of lesions such as crusts, papules. Ulcerations, scabs, erosion and areas of alopecia which grow to be very widespread on the horses' skin. They progressively began to lose appetite and eventually went off feeding for some time and could hardly walk.

Clinical examination: This was conducted by a visual evaluation and feeling the areas of the body that presented irregularities with the hand. The horses had alopecia lesion on the nostrils, left flank of the ventral lumbar vertebrae of the hindlimb and ball and socket joint of the femur. Dry crust lesion with areas of erosion were prominent on the radius and ulna region of all the animal sampled.

MATERIALNS AND METHOS

Four (4) horses were studied in this work. The clinical temperature, heart rate, respiratory rate and capillary refill time were examined as follows:

Clinical Temperature: Clinical temperature was obtained by the use of thermometer oiled with petroleum jelly. The horse's tail was raised while standing by the side and thermometer inserted into the anus and timed. The normal temperature was 37.5^0C to 38.6^0C for 60 seconds (Ohmura and Jones, 2017).

Heart Rate: was taken using a stethoscope. The stethoscope was placed on the left side of the thoracic cavity just behind the elbow and the heart beat was taken. Each echo of the heart was considered one beat. The normal heart rate is 32-36/min (Ohmura and Jones, 2017).

Respiration Rate: was taken by observing the movement of the ribcage of the horse in and out (an inhale and an exhale) .Stethoscope was used to listen to the breathing as the air travels across the trachea. The normal respiratory rate for adult horses is 12-18/min.

Capillary Refill Time: This was obtained by evaluating the mucus membrane or gum color. Normal gum is pink in color and moist on touch. Capillary refill time was tested by applying digital pressure with finger on the gum above the front incisor and removing it quickly. Time it takes for the gum to turn pink is known as the CPR. Usually, the CPR is about 2seconds. If longer, then there is a problem.

Clinical indices of the mucus membrane of the mouth were obtained by observing the color for anaemia while the nostrils were examined by visualizing the inner part of the nostrils for wetness

Faecal Sample Collection: Faecal samples were collected per rectum with a gloved hand into well-labeled sterile polythene bags and transported in ice packs to the laboratory of the Veterinary Parasitology and Entomology Department of the Michael Okpara University of Agriculture, Umudike for examination and identification (Esan *et al.*, 2018).

Examination of Fecal Samples

The test tube flotation technique was employed in analyzing the collected faecal samples.

The faecal samples were analyzed in the following manner: 2g of faecal sample was mixed thoroughly (using an applicator stick) with 2mls of normal saline solution in a test tube, the sample was then homogenized by stirring with the same applicator stick and the test tube filled to the brim with a saline solution having higher specific gravity than the organism. A cover slip was placed on the test tube and the test tube was placed inside a test tube rack undisturbed for about 15-20 minutes to allow eggs of lower density float to the surface of the saline solution and adhere to the cover slip. The cover slip was finally taken off the test tube, placed on a light microscope and viewed (Sanda *et al.,* 2019).

Blood Sample Collection: Blood sample collections was as described (Donovan and Brown, 1993).

The horse was restrained by lifting up the head while 22guage needle and syringe was pierce through ventrally via the jugular vein and 5ml of blood was drawn into the syringe and placed into a sterile Ethylene diaminetetraacetic acid (EDTA) bottle and taken to the laboratory for processing. The site of blood collection was sterilized using cotton wool soaked in methylated spirit.

Skin Scrapping Technique: Skin scrapping collections was conducted according to the procedures adopted by Chaya and Pande, (2007). A sterile scalpel blade was used on the area between the lesion and skin because the active organism is seen at the periphery of the lesion and the normal skin. The sample collected was deep enough to have contact with blood. The sample was then placed into a sterile sample bottle containing 2mls distilled water and taken to the laboratory for processing.

Sample Collection

Serum Biochemistry and Hematological Analysis: Blood sample collected was separated from the whole blood in a non-anticoagulant sample bottle (EDTA). The sample was centrifuged at 3000 rpm for 10 minutes as described by Yilmaz (2012), and the serum was stored in a freezer until ready for use. Analyses, including pack cell volume, total erythrocyte count, total leukocyte count and hematocrit value were determined using standard routine techniques. For differential counts, blood smears were prepared and stained with Giemsa (Jain, 1984). Total protein was determined by the Biuret method (Zaia et al., 1998), albumin by the bromcresol green method (Moreira, 2018). Alanine transferase (AST) activities by the colometric method of Reitman and Frankel,(1957) creatinine by the Sigma colorimetric method and Alkaline phosphatase (ALP) by the modified method of Bowers and McComb (Bower and McComb, 1966). The enzymes were measured at 37^0C and the results obtained presented in units per liter (Burtis and Ashwood, 1994).

Media Preparation**:** Potato Dextrose agar (PDA) and Sabouraud Dextrose agar (SDA) were the media used. The method described (Savitri-Sharma *et al.* 2010) was employed. A mixture of 65.0 grams of SDA in 1000ml/ distilled water were placed into a conical flask and shaken to make a suspension. Same process was applied for PDA. To the mixture, 2ml of gentamycin were added and mixed again using a sterile rod. The suspension was sterilized by autoclaving at 121^0C for15min at 15lbs'pressure. Solution was then allowed to cool at 56^0C before pouring into the petri-dishes and allowed to solidify.

Skin Scrapping: From the skin scrapping sample, a loopful was inoculated into Sabouruad Dextrose Agar and Potato Dextrose Agar respectively and incubated at room temperature for up to 14days. The sample was observed at different days of growth. From the cultured sample, needle mount technique as described (Barros *et al.* 2011) was carried out.

A loopful was picked from the culture and place on a sterile glass slide and thereafter a drop of acetone was placed on it and rocked properly. A drop of Lactophenol Cotton Blue was placed on a slide and subjected to microscopic view.

Biochemical test (Urease test): Urea agar was prepared according to manufacturer's instruction. Twenty grams (20g) of urea was dissolved in 100mls of distilled water. The mixture was sterilized by autoclaving at standard temperature and pressure. The agar was later poured into a bijou bottle and placed in a slanted position. To the urea agar slant, a loopful of the isolate was inoculated and incubated at room temperature for 24hr and thereafter for 48hr. It was observed that the positive reaction of color change to wine red was very slow.

RESULTS AND DISCUSSION

The horses had alopecia lesion on the nostrils, areas of alopecia on the left flank of the ventral lumbar vertebrae of the hind limb and ball and socket joint of the femur. Dry crusty lesions with erosion were prominent on the radius and ulna region. All the vital signs of the horses like heart rate and respiratory rate were higher than normal at 67/min and 15/min respectively, while the temperature was lower at 36^0C than the normal range as shown from table 1. Clinical examination shows that the mucus membrane of the mouth and nostrils was slightly pale and the capillary refill time was longer at >2/sec than normal of 0-2/sec. There was absence of any helminths as the faecal floatation method did not support the presence of any egg, larva or adult worm as all recoded <0 presences as seen from table 2. In the floatation technique, the solution use had higher specific gravity than the organisms to be floated so that the organisms rise to the top and the debris sinks to the bottom. The outcome of the hematological analysis of the horse shows that a packed cell volume (28%) was far lower than the values of normal horse and this is characteristics as shown in table 3. The differential white blood cell count table 4 of the horse shows that the test value of monocyte was 0% which is below the expected range of 0.5-7%. The serum biochemical analysis in table 5 showed that the aminotranferase (AST) was much higher at 612µ/l. The plates show the macroscopic view of the causative organisms at various days post incubation. Plate 1 show ulcerative lesions on the forelimb while Plate II shows patches of irregular lesion on the hindlimb which is generalized. The plate III shows the growth of the *Tichophyton verrucosum* at day 4 post incubation. The plate IV and plate 3 are growth stages of *T. verrucosum* at day 7 and day 14 respectively. The microscopic view of the organism is shown in plate V and plate VI.

Table 1: Clinical parameters of infected/diseased horses.

Rectal temperatures	37.5-38.6^{0}C	36^{0}C
Heart rate	32-36/min	67/min
Vital signs	Normal range	Test values
Respiratory rate	8-12/min	15/min
Capillary refill time	0-2/sec	>2/sec

Table 2: Faecal floatation of solid waste from horse

Parasite	Normal range	Test values
Egg	<0	<0
Larva	<0	<0
Adult worm	<0	<0

Table 3: Hematological evaluation of test sample from the horses

Indexes	**Normal range%**	**Test sample range**
Hb	11-19	13.6g/100ml
PCV	32-52	28%
Total WBC	5.5-12.5	8.55 ×103mm^3
Total RBC	6.5-12.5	8.3 × 10^{6}mm^3

Table 4: Differential white Blood cell count (%) of the horse.

Cells	**Normal range%**	**Test value**
Neutrophil	30-75%	58%
Basophil	0-3%	0%
Eosinophil	0-11%	4%
Lymphocyte	25-70%	38%
Monocyte	0.5-7%	0%

Equine Clinical Pathology, normal value for Horses. New Bolton center field service department

Table 5: Serum biochemical analysis of the horse

Factors/indexes	**Normal range**	**Test value**
AST	205-555	612µ/L
ALP	109-315	117µ/L
ATP	4.6-6.9	5.2g/dl
ALB	2.5-4.2	3.5g/dl
Total Bilirubin	0.1-0.9	0.8mg/dl
Creatinine	0.6-1.8	2.1mg/dl
BUN	8-27	29mg/dl

AST=Aminotransferase
ALB=albumin
TP= total protein

Pritchard, et al 2009

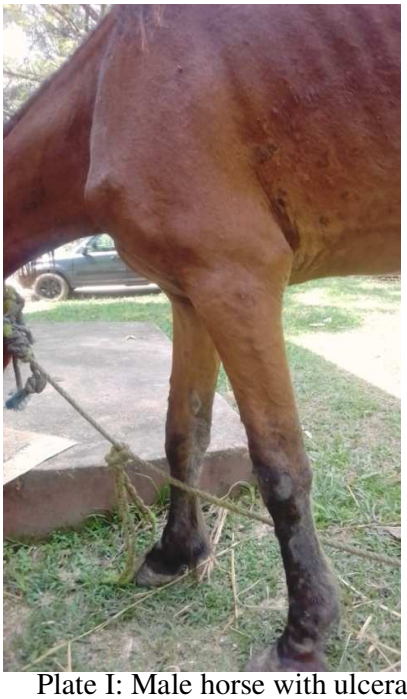


Plate I: Male horse with ulcerative lesion on the forelimb

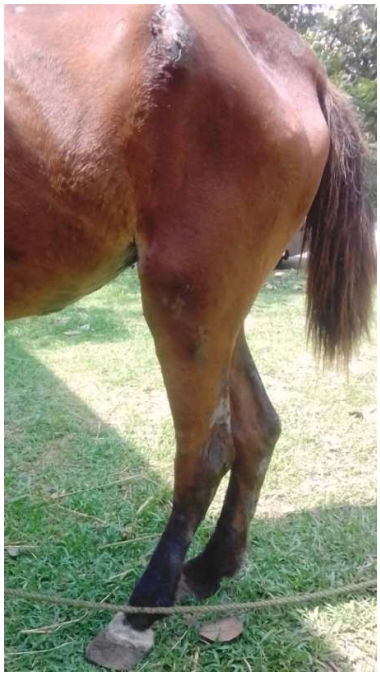


Plate II: Patches of Lesion on the hindlimb.

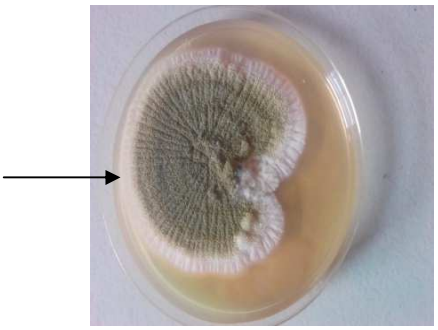
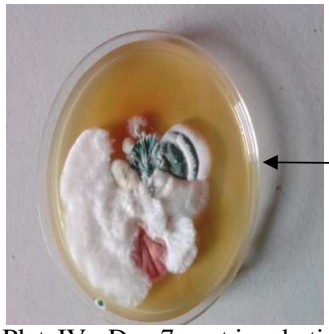


Plate III: Day 4 post incubation(SDA)



PlateIV : Day 7 post incubation (PDA)

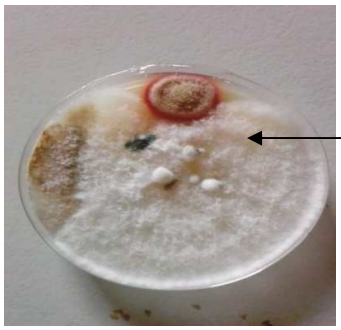


Plate V: Day 14 post incubation

The growth rate of Trichophyton *verrucosum* was slow in SDA but rapid in PDA. The texture was waxy, glabrous to cottony. From the front view, the colors were white, fluffy, folded, with central folding and red pigments at the edge, while the reverse is pale and slightly brownish at day 14

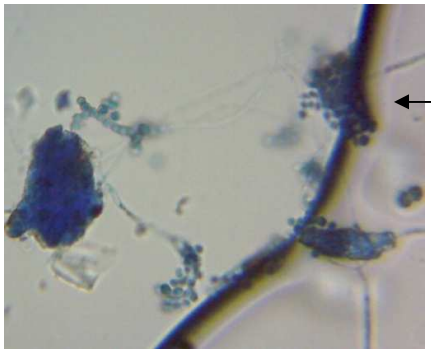


Plate VI : Saptate and hyphae ×40

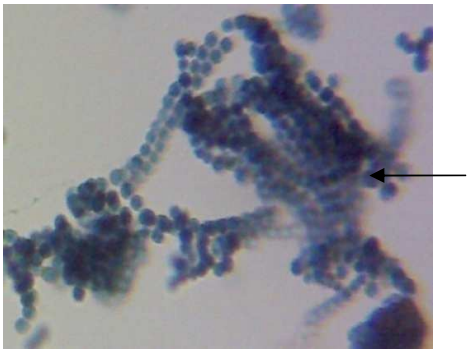


Plate VII: Chlamydospores of *T. verrucosum* ×400

Plate VI shows hyaline saptate, hyphae with microconidia, conadiospores while Plate VII shows multinucleated smooth, and spherical shaped Chlamydospores of T. *verrucosum* growing in chains at 37^{O}C and this observation as presented has been a remarkable feature used for T. *verrucosum* identification.

DISCUSSION

From the evaluation of the clinical parameters of the horses, the heart rate was higher than the normal heart beat of 32/min and this implies that the influence and effect of *T. verrucosum* affected the cardiac system thereby negatively causing distortion of the systolic and diastolic activity of the animal. This is a new understanding. Increase in heart rate may be due to metabolic, respiratory and heart disorder. Others could be stress, pain, disease and even exercise.

Heart rate is a useful indicator of the severity of the disease and its progression. The high heart rate as observed in this study is suggestive of terminal stages of severe disease and this is consistent with the report (Ohmura and Jones, 2017). The floatation method was used to examine the presence of eggs, larvae or adult worms since these could contribute to the emaciation and cachexic presentation of the horse. The presence of larvae may occur when larvae enter the skin causing it to itch and other clinical signs are those of pneumonia, cough, dyspnea and hemoptysis may mark the migration of larvae through the lungs. Though, Wakelin et al. (1996) reported that when heavy worm infestation is diagnosed, anorexia, fever, diarrhea, weight loss and anemia would result. However, the horse was free of all forms of worm either mature or immature form, hence not responsible for the emaciation.

The packed cell volume (PCV) of the horse (28%) was lower than the normal range of 32%-52%. This suggests that the causative agent or organism has a depleting effect on the blood or prevents the process of further production or hematopoiesis, the total red blood cell and white blood cell was within the normal range for healthy animal. Decreased pack cell volume generally means red blood cell loss from any variety of reasons ranging from cell destruction, blood loss and failure of bone marrow production. The possible causes of low PCV or anemia are ulcers, trauma, colon cancer, internal bleeding and drugs.(Charles and William, 2019).

The differential white blood cell count (%) was within the normal range of 0.5-7%, except monocyte which stood at 0%. Low monocyte directly exert microbial effects and this suggests that there is a disease in progress, since monocytes are involved in early defense against infections and this is in agreement (Yona *et al.,* 2013). Though the mechanism of infection may not be well understood, studies by Auffery et al. (2009) confirm that monocytes contribute to surveillance, wound healing and vascular endothelial growth.

The serum biochemical analysis was higher than the normal range of 205-555 µ/l for alanine transferase. Alanine transferase (AST) is one of the enzymes that help the liver convert food into energy. High level of this enzyme can be a sign that the liver is injured, irritated or infected, hence, the enzyme leaks out of the liver cells and this corresponds with the views of Rei, (1984). Besides alanine or amino transferase catalysing the distribution of nitrogen between amino acids, The primary clinical application of serum ALT measurement is the detection and differentia etiological diagnosis of hepatic disease as reported (Anach et al., 1981). The presence of a high AST suggests that the organism evaded the liver, heart and muscular walls of the heart. This is very significant because the two organs are responsible for the metabolic activities and physiologic activities of the horse. Any infection affecting these two organs could lead to a gradual decline in the health of the animal if not treated on time and subsequent death.

Also, the kidney had some defect as revealed by the slightly high creatinine and blood urea nitrogen. This makes it difficult for the kidney to effectively carry out ultra-filtration of fluid material. These fluids become toxic to the health of the animal if not processed and discharged as waste within the given period of time. Elevated creatinine level is known to be a strong predictor of mortality and this is in agreement with Castillon-Vela and Fermandez-Perez (2010). Castillon-Vela and Fermandez opinioned that horses from 1year to 5years, had creatinine as the independent predictors of mortality in multivariable analysis. Comparing patients with persistent elevated creatinine and those with normal creatinine level, it was observed that the mortality rate of 46.1% were recorded in elevated creatinine level as suggested by Aronson and Burger, (2010). The study highlights the fact that elevated

creatinine level is a strong predictor of Also, creatinine is a more reliable indicator of renal function than Blood urea nitrogen (BUN), because it is less influenced by other factors such as diet and hydration. Serum creatinine is increased by glomerular filtration, although both BUN and creatinine remain in the reference range. In most animals, 50-75% of renal function is lost and this is in agreement (Finco, 1989). Factors that may increase creatinine level are: necrosis or atrophy of skeletal muscles, infections, burns and fractures. Blood urea nitrogen (BUN) is formed by the liver and carried by the blood to the kidneys for excretion. Damaged or diseased kidneys cause BUN to accumulate in the blood as glomerular filtration rate (GFR) drops. Conditions such as heart failure, shock, bleeding into the gastrointestinal tract can cause BUN elevation and similar findings were described (Kirtane, 2005). Kirtane, suggested from his studies that BUN is the single best predictor of mortality followed by systolic blood pressure.

The macroscopic features of Trichophyton *spp* in the study coupled with the urease test which developed slowly were suggestive of the identification of *T. verrucosum. Trichophyton verrucosum* colonies appeared as colorless with a red pigment in the middle and this was similar to an earlier observation mortality in horses with heart failure. (Weitzmann and Summerbell, 1995). Though, there are some similarities in microscopic features of Trichophyton *species,* this isolate was distinguished from *T. equinum* whose culture presents a deep-yellow reverse with dark spot as reported (Amor *et al.* 2001; Ural *et al*., 2009). At a microscopic view, the arrangement of the chlamydoconidia is a feature used for identification of T. *verrucosum.* Trichophyton hydrolyses urea because they produce enzyme as virulence factors which hydrolysis urea and this is in agreement (Issa and Zangana, 2009 ; Weeks *et al.,* 2003). The horses may have contracted the infection from the environment, which could have been infected. The cattle and horses grazing together on the field within the premises of the university predispose the horse to *Trichophyton* infection and this is consistent with the findings (Quinn and Markey, 2003). Since transmission could be by direct and indirect means, during grazing, the horse usually lie down and scratch itself, hence, can easily pick the organism since it affects horses of all age and this is in agreement (Arstan *et al.,* 2007).

Conclusion: *Trichophyton verrucosum* was isolated and fully identified from the four horses grazing at Michael Okpara university farm.

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