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Epidemiological Studies and Molecular Diagnosis Compared with Bacteriological Examination for Detection Carriers of Strangles in Horses

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Abstract

Strangles is a highly contagious and serious infection of horses and other equidae caused by the bacteria *S. equi*. Strangles is characterized by abrupt onset of fever followed by upper nasal respiratory tract catarrh, as evidence by mucopurulent nasal discharge and acute swelling with subsequent abscess formation in submandibular and retropharyngeal lymph nodes. Our study showing prevalence of diseases in different age of horses and seasonal effect. Most dangerous Form of strangles is apparent healthy that shedding bacteria and causing infection to most susceptible one that can be detected by PCR.

Keywords: Streptoccous; PCR; Foals; Winter; Prevalence.

Introduction

Strangles is an infectious, transmissible worldwide disease of equidae. It continues to become one of the most significant respiratory diseases, wide spread distribution is favored by its highly contagious mode of spreads [1]. Strangles is caused by the bacterium *Streptococcus. equi* and is characterized by sever inflammation of the mucosa of upper respiratory tract, purulent thick creamy nasal discharge, enlargement of the head lymph gland forcing strangled breathing noises of the affected animal [2]. The disease may be introduced to a population by an animal incubating the disease, or by animal that has recovered clinically but was still shedding *S. equi* [3]. Indiscriminate use of antibiotics that are not strong or not administered for a long enough period to kill all the *Streptococcus. equi*, can render the disease dormant or can cause bastard strangles [4].

Bastard strangles occurs when there is spreading of the infection to sites other than the lymph nodes draining the throat as well as developing abdominal, lung and brain abscesses [5]. Prevention of strangles should involve the application of good control programme and detection of carriers which was help spreading the disease. At least 3 nasopharyngeal swabs are taken approximately weekly intervals from all recovered cases and their contacts and tested for *S.equi* by culture and PCR [6].

PCR assay is useful technique for direct detection of *S. equi* in clinical samples quick, reducing the time of diagnosis to one day and provided ability to detect organism even when become non-viable and used for detection healthy long term carriers [7]. PCR was optimal tool for screening of herds, testing of indi-

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vidual animal and obtained a definitive diagnosis during acute, chronic stage of illness and before antibodies are detectable.

In Egypt, the disease has become increasingly important as several outbreaks of strangles had been recorded on several horse breeding farms as well as individual horses, many authors reported the clinical picture of the disease, incidence, and immunization against the disease.

Poly merase chain reaction (PCR)

Timoney and Artiushin [8] Found that PCR test provided highly specific and sensitive results obtained more quick than culture, potentially reducing the time identify and isolate contagious or shedding horses.

Claire et al., [9] reported that PCR assay based on the use of degenerated primers in order to characterize an internal fragment representing approximately 85% of the genes encoding the manganese-dependent super oxide dismutase in various streptococcal type strains (*S. equi subsp. equi, s. equi subsp. zooepidemicus).*

Sechi et al., [10] used PCR-Ribotyping for identification *S. equi subspecies equi* strain isolated from an Ethiopian camel and compared the results obtained with those generated from two strains of the Pasteur Collection. The ribotyping showed the highest power of differentiation, distinguishing between the strains analyzed, whereas PCR- Ribotyping was able only to differentiate the camel isolate but not the strains from the Pasteur Collection. The application of this technique will be very useful to establish a clonal relationship among equine and camelids strains and help in prevention and cure of the equine and camel pathology.

Newton et al., [2] reported that PCR provided a potentially useful adjacent to culture of nasopharyngeal swabs in the detection of asymptomatic carriers of *S. equi* following out breaks of strangles in controlling a disease for isolation of a positive carriers. Al-Ghamdi et al., [11] used of repetitive sequence-based polymerase chain reaction for molecular epidemiologic analysis of *S. equi subspecies equi*. Laurent et al., [12] reported that amplified ribosomal DNA and restriction analysis was based on PCR amplification was recently to be a rapid and efficient method of identification *streptococcal* isolates. Alber et al., [7] used oligonucleotides primer in PCR designed according to species specific of the super oxide dis mutase A for identification and differentiation of *S. equi subspecies equi* and *S. equi subspecies zooepidemicus*.

Anzai et al., [13] studied that there is variation in the N-terminal region of an M-like protein of *S. equi* and evaluation of its potential as a tool in epidemiological studies during out break of strangles in using PCR test. Alber et al., [14] investigated that the presence of the super antigen encoding genes seeM and seeL by Polymerase Chain Reaction (PCR). During the test characterized *S. equi subsp. equi* strains from strains of various other animal pathogenic *streptococcal* species and subspecies. They found that the super antigenic toxins L and M seemed to be widely distributed virulence factors of *S. equi subsp. equi* and rare among *S. equi subsp. zooepidemicus* but did not occur among a number of other animal pathogenic streptococcal species. Gronbaek et al., [15] found that nested PCR represented a species specific and sensitive method for diagnosis of *S.equi* from clinical cases. It may, however, be desirable in future to develop detection methods with high diagnostic sensitivity and specificity without the potential problems inherent in PCR.

Materials & methods

Materials

Animals

A total number of 650 horses (400 of them were Arabian horses belonging to AL-Zahraa station. In addition to 250 horses from different farm in the shariakia government and horses admit to clinic in the Faculty of Vet. Medicine Zagazig University. Were employed to clinical examination and Epizootiology during the period from March 2005 – February 2006.

Samples

Samples for bacteriological examination:

1. Naso pharyngeal swabs

285 naso pharyngeal swabs were collected under complete hygienic condition from clinically affected animals with respiratory symptoms via ventral nasal meatus to the level of common pharynx and soft palate leading to the horse tries to swallow.

2. Lymphonde samples

60 lymph nodes samples 53 samples were collected from opened lymph nodes (48 samples from opened submandibular lymph nodes and 5 samples were collected from opened retropharyngeal lymph nodes and guttural pouch empyema) under aseptic condition after draining by introduction of swab into the abscessciated lymph nodes to obtain samples from the purulent exudates.

More over 7 lymphoid aspirates were collected, from the intact lymph nodes.

Samples for PCR

On the other hand 12 nasopharyngeal swaps were collected from apparent healthy animals (9 animals clinically improved after treatment and 3 in contact animals without history of strangles infection) were immersed in transport medium for PCR.

1- Taq DNA Polymerase

2- De-ionized water

3- Tris buffer (tris 10mM ,1mM EDTA PH 7.5)

4- Doxynucleotides Tris phosphate mixture (DNTPs)(1.25nM for every dATPDCTPdTTP and dGTP)

5- Taq Buffer with (NH4)2SO4 1.0 ml 25 mm $Mgcl_2$

6- Primers

primer sequence (5-3; forward and reverse)with size of fragement amplified in base pairs (nucleotide number in relation to SeM open reading frame) 325 (154-479)

F 5 CATACCTATCTCCATCAGCAATCCG

R 3 CGAACTCTGAGGTTAGTCGTACCGG

7- Agarose gell

8- Loading buffer 11: 250 ul 0.01% bromophenol blue.

Biotechnology Department Bio Basic Inc. unit 11-12, 315 Steel case Road east Mark hamintorio L3R2R5 Canada.

Methods

Clinical examination

650 horses were examined clinically according to Kelly [16] with special attention to body temperature, nasal discharge, lymphonde of the head and neck region.

The nasal discharge was examined to declare if it is unilateral or bilateral, continuous, or intermittent, scanty or copious, serous, mucoid, mucopurulent or purulent.

The lymphondes of the head and neck region were examined by inspection & palpation.

Bacteriological examination

1- 285 Sterile swabs were used to take the samples from horses showing respiratory symptoms from nasal discharge and 60 samples from abscessated lymphnodes.

2- All collected samples were immersed in transport medium and transported on ice bag to the laboratory of faculty of Vet. Medicine Zagazig University.

3- Direct smear stained with Gram, s stain for morphological identification followed by direct microscopical examination. secondly streaked on 5-7% sheep blood agar or 5-7% horse blood agar and Brain heart infusion agar media.

4- The suspected streptococcal isolates were biochemically identified by the method recommended by Mackie & MacCartteny [17] of sugars fermentation (lactose, trehlose, mannitol, salicin, sorbitol) containing bromocresol purple as indicator, the acid production by sugar fermentation was detected by the change in color into yellow .

5- Serological identification for confirmation for Latex grouping reaction Facklam and Carey

[18] is based on group specific components of the cell surface which can be extracted from the cell wall and identified using immunological procedures.

Polymerase chain reaction (PCR): According to Timoney and Artiushin [8]

Samples Extraction:

1- Nasopharyngeal swab fluid was collected by squeezing the swab between sterile forceps.

2- Swab fluid or lavage (500 ul) were centrifuged at 10.000g for 5 minutes.

3- The deposit was re suspended in 1xGene Amp BufferII[6], 0.5% (V/V) Tween 20 and 100 ug /ml Proteinase K[1]; 25 ul was generally used but if a large deposit was produced, sufficient was added to at least 2 times the volume pellet.

4- The samples were incubated at 55° c for 30 minutes, boiled for 5 minutes and centrifuged at 10,000 g for 5minutes to collect the supernatant fraction for immediate PCR.

5- Aliquots of the original samples were stored at -20^{0}c.

6- PCR-3 was conducted by mixing 5ul of samples with 2.5 ul 10 x Gene Amp PCR buffer, 0.5ul 10 mmol /I dNTPmix,

7- 1ul each first round primer at 25 pmol/ul, 0.1ml AmpliTaq Gold 5 units/ul, 14.9 ul of water and 30 ul light mineral oil for PCR.

8- The thermocycling condition for the first round of the reaction were 95^0 c for 10 minutes followed by 30 cycles of 95 ^{0}c for 101min, 60 ^{0}c for 1 min, 72^0 c for 1min 30 s followed by a period of 5min at 72^0 c .

9- The second round of the reaction was conducted in the same tube as the first round without disturbing the top mineral oil layer.

10- The second round reaction mixture, centrifuged from the top of the tube, contained 5ul 10 x Gene Amp PCR buffer, 1ul 10 mmol/I dNTPmix, 2ul each second round primer at 25pmol/ul, 0.2ul AmpliTaq Gold (5units /ul) and 14.8ul water (12.8 ul for PCR-2).

11- The thermo regulating conditions of the second round were 95^0 c for 1min, 65 c for 1min, 72^0 c for 1min followed by a period of 5 min at 72^0 c.

Detection of PCR products

1- PCR products were detected by electrophoresis of 3ul of sample mixed with 3ul sample buffer in 2% (w/v) agarose[8], 0.04mol /I Tris acetate (ph 8.3), 0.001 mol/I ethylene diamine tetra acetic acid[1] in GNA 100 appartus[9] at 115V for 45 min.

2- Product sizes were determined by comparison with relative mobilities of the Gibco 1 kb standards[10].

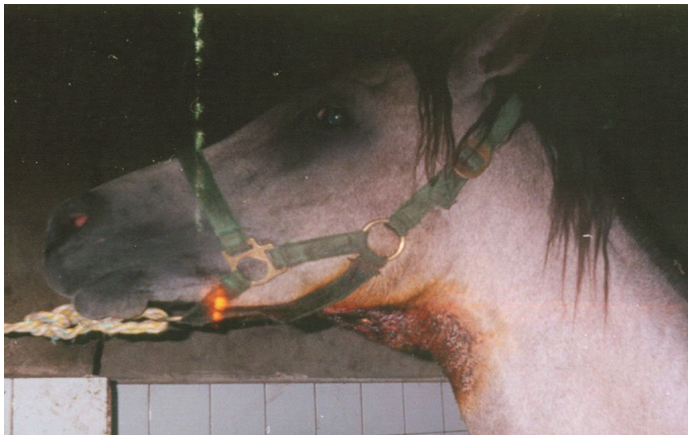
3- Gels were examined on a UV trans illuminator after immersion in 0.5ug/ml ethidium bromide for 15 min.

Result

Clinical examination

During the period from March 2005 - February 2006 examination of 650 horse showed that 134 animals were suffered from upper respiratory tract affection out of them 51 with typical clinical signs of strangles (Picture 1) marked systemic reaction, bilateral or unilateral purulent nasal discharge. enlargement, painful swelling and abscessation submandibular lymph nodes, moist cough while 76 showing mild respiratory signs, in other hand 7 cases with complicated strangles (Picture 2) but most dangerous one is carriers in 7 apparent healthy animals examined bacteriologically and PCR. All strangles forms shown in Figure 1.

Picture 1: A case of strangles showing mucopurrlent nasal discharge.



Picture 2: A horse suffering from Strangles showing swollen submandibular lymph nodes.

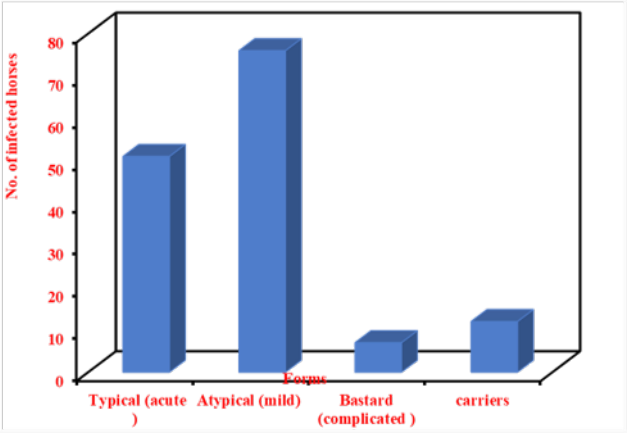


Figure 1: Forms and clinical signs of strangles in horses.

Epidemiological studies

Epidemiological findings studied all animals for Cumulative Incidence (CI), age susceptibility relationship, and seasonal occurrence of the disease according to Martin et al, [19].

No. of animals developing a disease during a time period

I- CI = ...

No. of animals at risk at beginning of that period

The cumulative incidence of strangles at Al – Zahraa station during the period of March 2005 to February 2006 was 0.215 where 86 horses of 400 were infected.

while cumulative incidence of strangles in sharkia government and horses admitted to the clinic of fac of Vet. Med. during that period was 0.192 where 48 out of 250 animals examined were infected and the total C I was 0.0206 where 134 out of 650 examined horses were infected as shown in Table 1 and Figure 2.

II. **Age-susceptibility relationship**

The infection rate in age group less than 1 years was 16.36 % where 36 out of 220 examined horses were infected with S. equi subspecies equi. In group of 1-3 years, 87 out of 305 horses were infected with S. equi subspecies equi representing 28.5 % with significant difference in comparison with other ages groups. In age group of more than 3 years were infected with S. equi subspecies equi representing 8.8 % where 11 out of 125 animals were proved to infected with S.equi subspecies equi. The obtained result are recorded in Table 2 and illustrated in Figure 3.

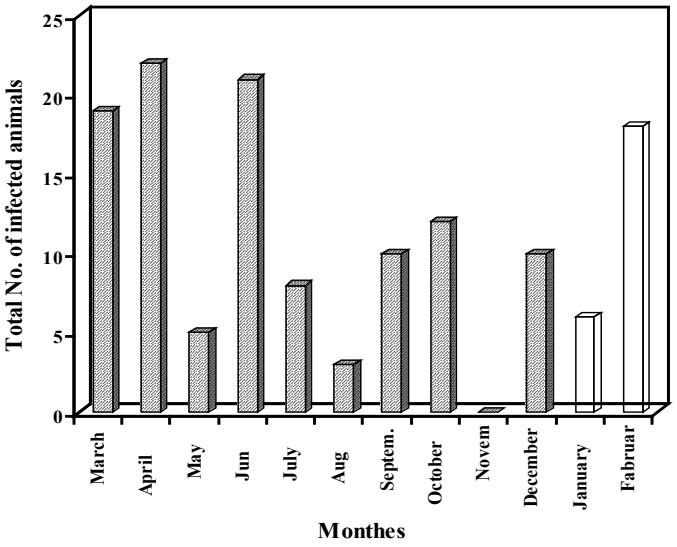


Figure 2: The cumulative incidence of strangles in horses during March 2005, February 2006.

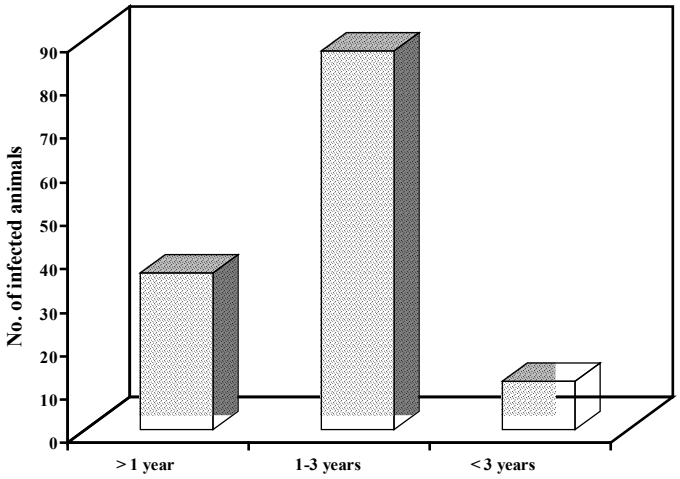


Figure 3: Age –susceptibility relation ship of strangles in horses during March 2005, February 2006.

III-**Seasonal dynamic of strangles**

The infection rate of strangles was higher in spring season than other seasons where 61 out of 650 horses representing 45.52%, followed by incidence rate of 25.37 % in winter season (34 infected horses out of 650) then the incidence was 17.16 % in summer months (23 out of 650). The lowest infection rate was recorded during autumn months 16 out of 650 were infected representing 11.94 % as shown in Table 3 and Figure 4.

IV. The period of shedding of s. equi subspecies equi from recovered horses

Weekly bacteriological examination of nasopharyngeal swabs collected from clinically recovered animals ranged from more than 3 weeks up to 8 months in 9 cases of Arabian horses at Al zahraa station, and these animals were examined using PCR.

Results of bacteriological examination

Bacteriological examinations of the collected samples re-

vealed that presence of *Streptococcus equi subsp. equi* either alone in a pure pus collected from closed lymph nodes under complete a specific condition and mixed with other Grams positive bacteria of genus streptococci and unidentified r bacteria either in pus of opened lymph nodes and nasopharyngeal swabs by direct Microscopical examination showed Gram positive cocci arranged in long chains picture (3). On Blood agar media were translucent, mucoid with clear zone of hemolysis. And the resultant colonies on tryptone soya agar media were mucoid and like dew drop appearance.

Trials for detection of carrier animals by bacteriological examination and PCR

Nasopharyngeal swabs were collected from 12 animals (9 clinically recovered horses after treatment in addition to 3 in contact animals without history of previous infection) up to 11 months for detection of carriers.

On Bacteriological examination one sample (8.33%) positive culturing for 1st week, one sample (8.33%) positive culturing for 5th week following recovery, one sampling (8.33%) positive for 6 month, 2 samples (16.7%) positive at 7th month and 2 samples (16.7%) positive up to 8 month.

Variable shedding of microorganisms (intermittent), these indicate apparently healthy animals still persist of shedding *S. equi* from 1st weeks (8.33%) up to 8 months (16.7%). Furthermore, all samples (1-9) are negative culturing for 2 successive months (9 and 10 months) following recovery while samples 10, 11, 12 all are negative culturing then all samples were examined by PCR. By application of PCR techniques one strain amplified 325-bp fragments of 12 horse nasopharyngeal swab samples identified as s. equisub spp. equi.

Results of PCR indicated persisted shedding of *S. equi* up to 11 months following recovery as on samples (1,2,3,6,10,11,12) of 7 (58.3%) out of 12 nasopharyngeal swapping were positive indicated that importance of application PCR.

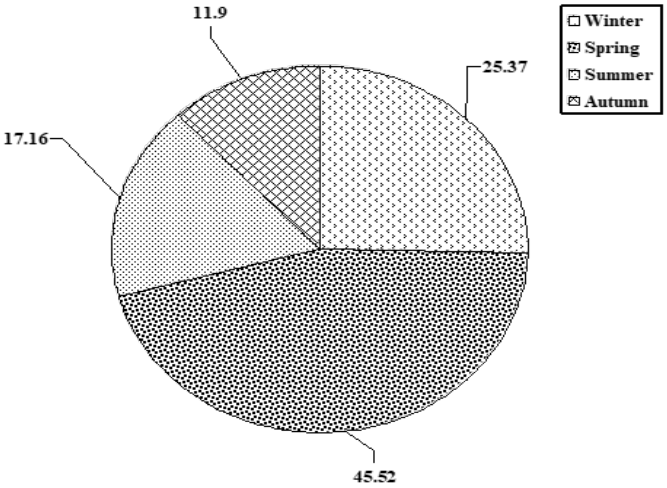
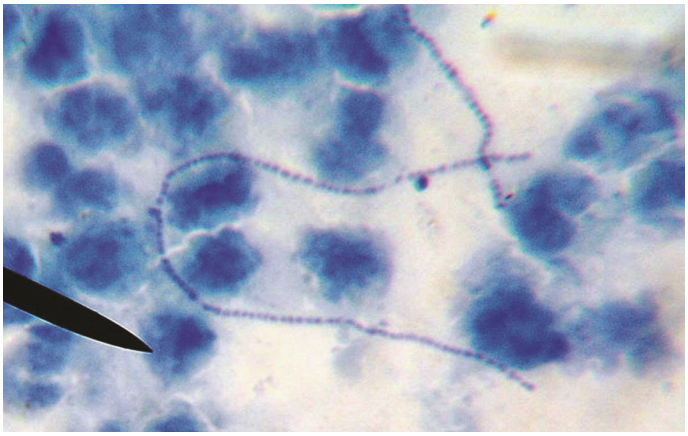
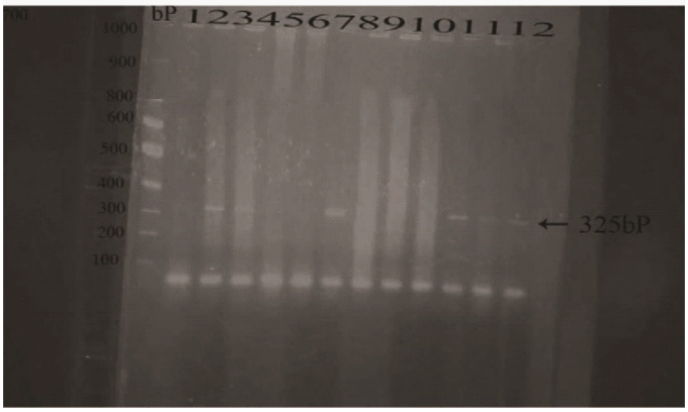


Figure 4: Seasonal dynamic of strangles in horses during March 2005, February 2006.



Picture 3: S.equi subspecies equi in asmear of pus from acase of strangles showing Gram–positive cocci arranged in long chain (Jene, s modificatication of Gram stain).



Picture 4: Detection of S.equi DNA in nasopharyngeal swap for carrier of Strangles by PCR.

Table 1: The cumulative incidence of strangles in horses during March 2005, February 2006.

Month	No. of new cases in al Zahraa –station	CI in Al Zahraa – station (%)	No. of new cases in sharikia government	CI in sharikia overnement (%)	Total No. of infected animals	Total CI (%)
March	7	1.7	12	5 .04	19	2.9
April	8	2.04	14	5.9	22	3.3
May	5	1.26	0	0	5	0.76
Jun	12	3.09	9	3.73	21	3.2
July	8	2.04	0	0	8	1.23
Aug	0	0	3	1.09	3	0.46
Septem.	10	2.56	0	0	10	1.5
October	7	1.7	5	2.04	12	1.8

Novem	0	0	0	0	0	0
December	2	0.5	8	3.3	10	1.5
January	6	1.5	0	0	6	0.9
February	18	4.7	0	0	18	2.7

Table 2: Age susceptibility relation ship of strangles in horses during March 2005, February 2006.

Cases Age	No. of examined animals	No .of infected animals	Percentage of infected animals
> 1 year	220	36	16 .36%
1-3 years	305	87	28 .5 %
< 3 years	125	11	8.8 %
Total	650	134	20. 6%

Table 3: Seasonal dynamic of strangles in horses during March 2005, February 2006.

Cases seasons	No. of infected animals	Percentage (%)
Winter	34	25.37
Spring	61	45.52
Summer	23	17.16
Autumn	16	11.9
Total	134	20.6

Discussion

The name strangles was coined because affected horses sometimes suffocated by enlarged lymph nodes that obstructed the air ways [6].

Concerning the epidemiology of the disease, the obtained results revealed that the cumulative incidence was 20.6% during the period of from March 2005 to February 2006, such relative high incidence may be attributed to the nature of management of most examined animals especially those at AL-Zahraa- station, the population of large numbers and the prescience of a source of infection facilitated the spread of infection resulting in high incidence.

Similar results were reported by Hunger Ford [20] who stated that the morbidity rate of strangles might be as 10% but up to 100% and the same conclusion was reported by Fallon [21], McGee (1969) and Radositis et al. [22]. While Ford and Lokai [23] found that the morbidity rate of strangles ranged from 30 to 100%.

Concerning the age susceptibility relationship, it was found that the higher susceptibility was in age group of 1-3years may attributed to the loss of passive maternal immunity which protect the younger from infection, and also the probability of exposure of such age group to the infection is higher through the contact during training, showing and stress by working.

In age group of more than 3 years the infection rate may relatively lower due to the natural immunity which was acquired after natural infection that mostly occurred by the time, they are less than 3 years of age.

These results were agreement with Baker [24] who reported that strangles was a disease of growing horses (less than 3years old) also coincided with Galan et al. [25] who reported that antibodies for strangles were passed from previously infected mares to foal via colostrum and were secreted into the foals nasopharyngeal and upper respiratory tract mucosa during the first few months of lives and protected them during this short period.

Also, agreement with Hafez et al. [26] concluded that all ages of horses contracted the disease and the age specific morbidity rates of strangles during this study and the age specific morbidity rates of strangles in different age groups were 48% in1-3 years, 12.66% in less than 1 years and 10.4% in horse more than 3 years of age.

Concerning the seasonal dynamic of strangles it was found that the disease occurred in all the seasons of the year, but the higher incidence was in spring (45.52%), while lowest rate at autumn (11.94%). There was significant difference between seasons may be due to exposure to stress factors in spring like relative humidity, poor nutrition, over working and overcrowding in addition to the availability of source of infection. This agree with Baker [24], Hunger Ford [20] who stated that bad climatic, bad hygienic, control and prophylactic measures in stud played a great predisposing role in starting and propagation of out breaks of strangles.

The obtained results in agree with Ebid et al [27] who reported that higher incidence of strangles in spring and on the other side Radositis et al [22] stated that strangles is most likely to occur in cold and wet weather.

In addition to PCR can detect a single molecules of target DNA in a test samples by the production of millions of copies laying between oligonucleotides primers, also PCR can detect a single organism amongst millions of others and detecting DNA from dead organisms that in most environments are usually much more numerous than living bacteria. PCR much more sensitive than culture at presumptively identifying animals that may be a symptomatic carriers of *S. equi* the identification is presumptive in that is based on the detection of DNA from either live or dead bacteria.

Sweeny et al. [28] reported that prolonged asymptomatic *S. equi* infections using standard bacteriological culture techniques [29] and these coincided by Newton et al. [30] that found 3 consecutive negative swabs by culture were not always sufficient to detect *S. equi* carriers and these illustrated that the intermittent recovery of *S. equi* from nasopharyngeal swabs in these horses, was due to persistent infection in one or both guttural pouches which escaped only irregularly into the nasopharynx. Details of strangles control strategies have been made by several authors Yelle [31], Sweeny (1996) and Timoney [32]. The overall aims of the control measures were (i) to limit transmission of *S. equi* between horses by direct contact or indirectly via fomites, personnel environment (ii) to identify *S. equi* carriers when horse become a symptomatic.

PCR must be used alongside culture of nasopharyngeal swabs as a preliminary step to identify a large proportion of horses that continued to carry *S. equi* after clinical signs had disappeared.

Ethical Animal Research: This study has been done in accordance with the principles and guidelines of animal care and use with the help of veterinarians of El Zahraa station and faculty of veterinary medicine, Zagazig University, Egypt.

Owner confirmed consent: Applicable at Zagzig university. Faculty of veterinary medicine.

Data accessibility statement: The data that support the findings and this studies are available from the corresponding author upon responsible request

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Author contribution

A. Hassenin contributed to study design and implantation, data analysis, manuscript preparation fikray H. Yousef and Abdellah abou zeid gives direction for preparation and review manuscripts.

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