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RESEARCH ARTICLE

Used of Real -Time PCR technique for detection of Streptococcus equi from horses infected by strangles in AL – Diwanyia Provence, Iraq

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Abstract

Real-Time PCR technique is specific and accurate molecular diagnostic technique that used in detection of microorganism based amplification of specific gene sequence in target organism. In this study Real-Time PCR technique based syber green dye amplification were used for specific confirmative detection of streptococcus equi isolates that collected form nasal chambers of infected horse by Strangles. The results was shown (24/30) positive isolates at percent (80%). This study was concluded that Real-Time PCR technique is easy and specific technique use in specific diagnosis of Streptococcus equi more than clinical and bacteriological examination.

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Introduction

Strangles is caused by bacterial infection with Streptococcus equi subspecies equi referred to as S equi, the agent of strangles. These streptococci are beta hemolytic and belong to Lancefield group C. (1,12) The bacteria typically infect the upper air way and lymph nodes of the head and neck, the disease has been in the equine populations, particularly affecting young horses, and recur on farms with previous outbreaks of the disease. Strangles was described in early veterinary science literature and first reported by Jordanus Ruffus in 1251 (2,13). It is one of the most commonly diagnosed contagious diseases of the horse , world wide .The persistence of this infection on farms is multi – factorial (3,14). The bacteria can survive in water sources for over a month , but the primary source of recurrent infections is most likely asymptomatic carrier horses that can shed the bacteria to other horses for months to years. (4,15) A recent study using polymerase chain reaction (PCR) diagnosis of nasal swabs showed that a symptomatic S . equi carries were positive , PCR can be used as a tool to help control the spread of strangles by diagnosing infected animals including carriers that standard culture methods may fail to identify . The use of PCR to evaluate nasal – swab samples for S . equi has been previously described by investigators in Europe (5 , 6) .

Materials and Methods

Samples collection

A 30 Samples were collected from nasal cavity of clinically infected horse with strangle at period from January to March in different local field in Diwanyia city, by using sterile cotton swabs. Then the samples transport to laboratory for bacterial isolation.

Bacterial isolation and identification

For isolated of streptococcus equi, nasal swabs samples were cultured on blood agar plates and incubated an aerobically at 37C for 24 hours (7). After that the B-hemolytic streptococcus growing colonies were identified by gram stain method and biochemical characteristics (8). The biochemical tests to be carried out were includes, catalase reaction sugar fermentation tests of (Trehalose , lactose , Mainitol , salicin and Maltose) (9).

Bacterial genomic DNA extraction

To performing the Real-Time PCR technique, bacterial genomic DNA was extracted from 1ml of streptococcus equi isolates inoculated in overnight incubation Brain Herat Infusion Broth, by using (Presto^{TM} Mini gDNA Bacteria

Kit, Geneaid. USA). Where, the extraction was done according to company instruction. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, the store in -20C at refrigerator until perform Real-Time PCR.

Real-Time PCR

Real-Time PCR technique was performed for specific amplification of conserved region in M- protein gene (seM) that responsible for detection of in Streptococcus equi bacterium. This technique was carried out according to method described by Lindahl S.(2013). The Primers for SeM gene of S. equi were design in this study from the SeM gene sequence of Streptococcus equi subsp. equi strain SB 77/10 M protein (seM) gene, GenBank code (JX502611.1) by using NCBI GenBank Database and the last version of Primer3 plus design online, then the primers were provided by Bioneer company. Korea as showed in following table (1):

The Real-Time PCR amplification reaction was done by using (Accupower^{TM} 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table (2):

These qPCR master mix reaction components that mentioned in table was placed in sterile white qPCR strip tubes and transferred into Exispin vortex centrifuge for 3minutes, the place in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following figure:

Results and Discussion

Diagnosis showed that in apppulation of 30 horses on different farms with endemic strangles , in AL – Diwanyia city , (24 horses were positive for S. equi . There were all positive cases (24 horses) that were showing clinical signs of active S. equi at Real – Time PCR testing – standard bacterial culture methods yielded appositive diagnosis for only 1o infected horses .

Primer	Sequence		Amplicon
seM	F	GCCTCTGTTGGGGATTTACA	185bp
	R	GCAATCCGCTTTTCAATTTC	

qPCR master mix	Volume
Bacterial gDNA template	2.5µL
2X Green star master mix	25µL
seM Forward primer (10pmol)	1µL
seM Reverse primer(10pmol)	1µL
DEPC water	20.5µL
Total volume	50µL

qPCR step	**Temperature**	**Time**	**Repeat cycle**
Initial Denaturation	95 °C	3 minute	1

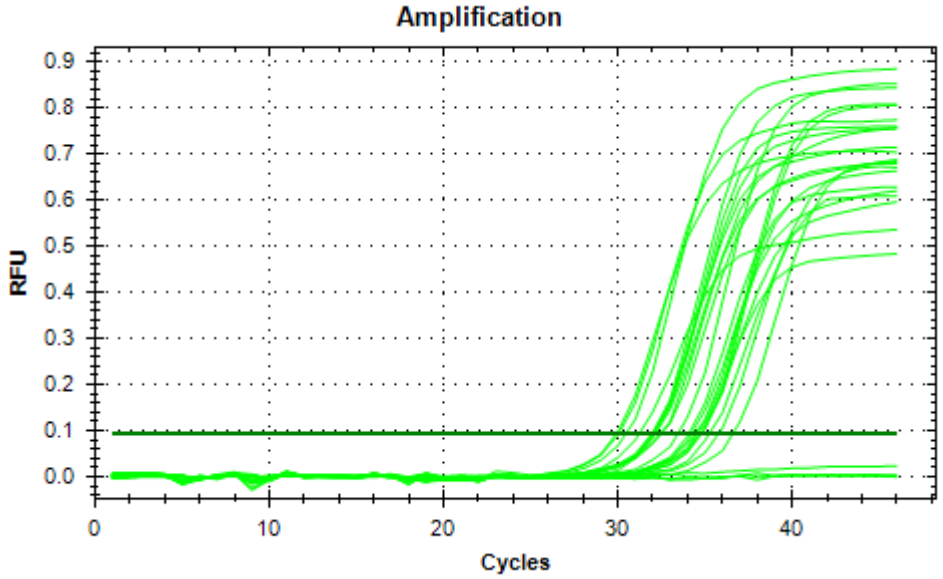
Denaturation	95 °C	10 sec	45
Annealing\ Extension	60 °C	30 sec	
Detection(scan)			
Melting	60-95°C	0.5 sec	1

Table (3) sugar fermentation by S. equi

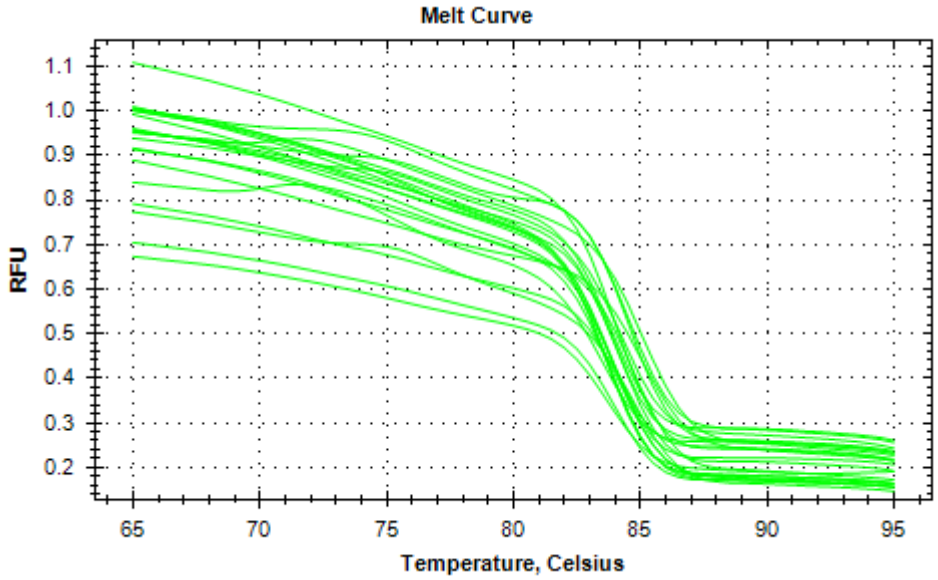
Mathose	Trehalose	Sorbitol	lactose
-	-	-	+

Sugar ferment that indication for S. equi group c .

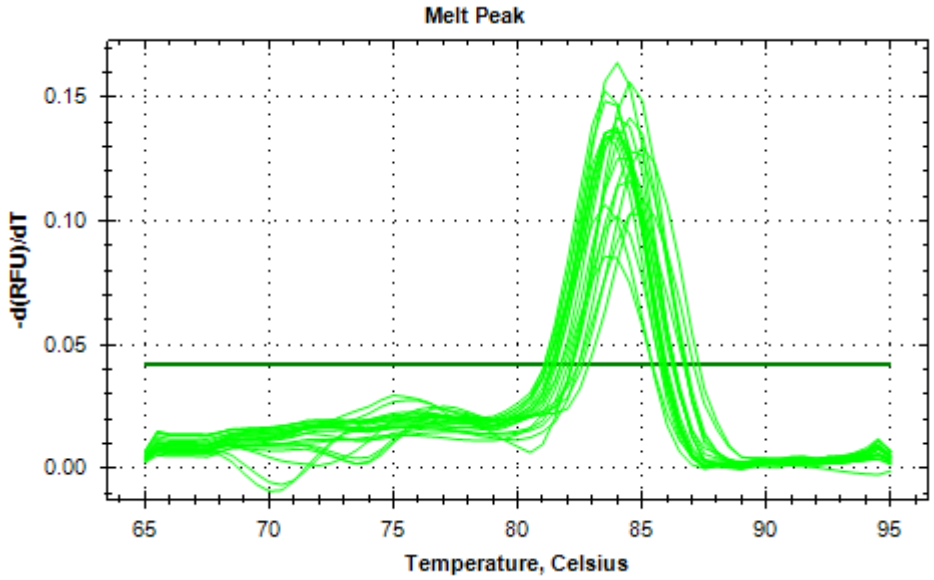
Real-Time PCR assay for detection of Streptococcus equi were shown specific direct detection out from (24/30) from nasal samples of horse with respiratory infection signs. The specificity of sem gene primers that amplification by SYBR Green dye based Real-Time PCR was determined by dissociation curve (Melt Curve). Where the positive amplification product samples show specific amplification at melt peak mainly at (Tm: 80C°) without primer diamer or nonspecific products. (Figure-1,2,3)



(Fig. 1): Real-Time PCR amplification plots that shown from the (29 to 36 cycles). The samples with amplification appeared at 29 cycles contained large amount of DNA while the samples with the amplification appeared at 36 cycles contained lower quantity of DNA for Str. equi, so the amplification appeared later, samples were negative which appeared under threshold line.



(Fig. 2): Real-Time PCR Melt curve for Str. equi seM gene positive samples.



(Fig. 3): Real-Time PCR Melt peak that shows the melting point for Str. equi seM gene ranged from 82.5°C to 84.5°C for positive samples.

Well	Fluor	Content	Sample	End RFU	Call
H06	SYBR	Unkn	Str. equi	0.0223	
H05	SYBR	Unkn	Str. equi	0.410	(+) Positive
H04	SYBR	Unkn	Str. equi	0.479	(+) Positive
H03	SYBR	Unkn	Str. equi	0.0619	
H02	SYBR	Unkn	Str. equi	0.472	(+) Positive
H01	SYBR	Unkn	Str. equi	0.340	(+) Positive
G06	SYBR	Unkn	Str. equi	0.00424	
G05	SYBR	Unkn	Str. equi	0.841	(+) Positive
G04	SYBR	Unkn	Str. equi	0.500	(+) Positive
G03	SYBR	Unkn	Str. equi	0.772	(+) Positive
G02	SYBR	Unkn	Str. equi	0.411	(+) Positive
G01	SYBR	Unkn	Str. equi	0.00487	
F06	SYBR	Unkn	Str. equi	0.00172	
F05	SYBR	Unkn	Str. equi	0.847	(+) Positive
F04	SYBR	Unkn	Str. equi	0.614	(+) Positive
F03	SYBR	Unkn	Str. equi	0.00756	
F02	SYBR	Unkn	Str. equi	0.624	(+) Positive
F01	SYBR	Unkn	Str. equi	0.461	(+) Positive
E06	SYBR	Unkn	Str. equi	0.00206	
E05	SYBR	Unkn	Str. equi	0.759	(+) Positive
E04	SYBR	Unkn	Str. equi	0.705	(+) Positive
E03	SYBR	Unkn	Str. equi	0.655	(+) Positive
E02	SYBR	Unkn	Str. equi	0.676	(+) Positive
E01	SYBR	Unkn	Str. equi	0.607	(+) Positive
D06	SYBR	Unkn	Str. equi	0.582	(+) Positive
D05	SYBR	Unkn	Str. equi	0.881	(+) Positive
D04	SYBR	Unkn	Str. equi	0.744	(+) Positive
D03	SYBR	Unkn	Str. equi	0.355	(+) Positive

(Fig. 2): Real-Time PCR end point amplification positive results of seM gene in Streptococcus equi in horse.

The DNA of which is almost identical to that of S. equi (9 , 10) Results from other studies shown that PCR is least three times more sensitive than bacterial culturing for detection of S. equi in samples obtained from nasal swabs (1) , but PCR testing does not confirm active infection , therefore , positive PCR samples should be confirmed with bacterial culture (11) . in our study all positive cause 80% (24 horses) were positive for S. equi at Real – Time PCR testing this disagree with Pobert (2006) . 25 , 5 % . Thus Real Time PCR is afar superior tool for screening a herd for S. equi to identify individual horses for treatment and control .

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