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Real Time PCR Compared with Microscopical Examination of Larva Culture for Strongylosis Diagnosis in Horses

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Abstract

Strongylus vulgaris was common in equine during the past few decades So far, routine daily uses of anthelmintic causing resistant and more shedding. So, asymptomatic cases of internal nematodes causing environmental contamination by excretion more eggs and worms. Apparently goal of our study, comparative test for real time PCR with larva culture techniques and prevalence of this parasite in American horses Current studies done in 110 horses were diagnosed for nematodes with molecular assay and compared with regular microscopic exam, out of them 26 horses went through routine examination for comparison and detection continues shedding of egg for variability efficacy for both diagnostic assay.

Keywords: Strongylosis; Horses; Strongylus Vulgaris; Larva; Fecal Samples; PCR

Introduction

Huge abdominal crisis and losses in horse population due to sever infestation with internal nematodes specially strongylus vulgaris. Pathagnomic lesion of strongylus vulgaris accompanying migration larvae to internal organ and blood vessels [1]. Strongylosis most common internal parasitic diseases affecting more than 80% equine population all over the world [2]. Cyathostomins is one of the small strongyle, are highly important internal parasites of horses mostly 90% infected by more common species of nematodes [3,4]. The adult worm of nematodes infections with high rate 60% [5,6]. The highly prevalence of infection and most equine e.g donkey, horses likely to get out of the infection at end of their life [7,8]. Large number of strongyles infestation causing inflammatory enteropathy reduces intestinal motility, colic [9]. Clinical symptoms of larvae infestation casing lethargy, emaciation, general weakness, severe anemia, colic and thrombosis [10].

Materials & Methods

Faecal Samples

Generally, 110 fecal samples from Horse entered to veterinary hospital were gathered from Jan 2018 to Sept 2018. Rochester, USA. Stoll's method for count egg per gram fecal samples and significantly all samples containing more than 15 egg per gram feces was kept for further examination.

Let say that a USA horses count about 2.3million with a possibility for infestation with internal parasites of 2% [11-31] at least 110 horses was used in this study Group of horses are moving outside for nature exposure to infection while grazing on day 45 with same infestation of S. vulgaris species all same arrangement of feeding facilities, water, and all other supplies in same daily basis. By 45 day arranged group depend on sex separation box to detect. Group of same sex, on the top basis of antigen antibody detection was haphazard treated and classified into three groups: (1) group

1: once daily used as control group (2) group 2: once daily or (3) group 3: divided pasture for each group separately with same size and supplies. All group stayed in their assigned In 26 of the 110 investigated horses, follow-up examinations were carried out 4–6 months after the initial examination., For sampling , a total of 26 samples of the 43 from variable locations and different environment taken for analyzed samples with molecular techniques and stoll's method and their compared results shown in (Table 1) .

Horse ID	1	2	3	4
Initial detection Dates of follow-up examinations	05/27/2018 09/16/2018	06/04/2018 10/30/2018 10/31/2018 11/01/208 11/02/2018 11/03/2018 11/04/2018 11/05/2018	08/06/2018 02/11/2018 02/12/2018 02/13/2018 02/14/2018 02/15/2018 02/16/2018 02/17/2018 02/18/2018 02/19/2018 02/20/2018 02/21/2018	06/13/2018 02/07/2018 02/08/2018

Table 1: Routine Examination for horses cases positive PCR.

Egg Detection, Concentration and Isolation

By 10th day fecal samples from each horse for separation protein specific antigen assay. Centrifugation for samples recorded in old studies, to different layers of fluid above surface and [32]. Using mac master kits of fecal analysis according protocol found in manufactures.

Dilution for each fecal sample using aliquots and buffer for counting to help separation debris from other content analysis with variation of isolated larva shown with significant value function was used to detect antigen antibody response of sampling reaction.

Eggs have been washed by sterile saline, and then put into a sterile cup and into 2ml aliquots, for concentration and remove debris. Floating solution was taken away into sterile Eppendorf tube for condensation and kept in deep freezing until used.

Stoll's Method

Fecal samples kept moisturized to help growth of egg and hatching from L1 up to L3 using funnel, good venti led room so can prevent any dryness, to see motility of larva traveling from clear cloth to funnel to be picked up by forceps examined under microscope to identify different stages [17].

Genetic Specific Protein Isolation from Fecal Samples

Added 150 fecal samples from each animal separately either diseases of gastric illness placed in clean refrigerated cup to be examined as quickly as possible. Cup containing glass beads to help separation different layer of fecal samples recognized by denaturation to be genetic acceptable for DNA isolation with adding different solution that increase density and capacity. For all samples put in centrifuge to be separated into different layers with adding specific buffer for antigen reaction.

DNA Extraction from Isolated Eggs

Added 150 fecal samples from each animal separately either diseases of gastric illness placed in clean refrigerated cup to be examined as quickly as possible. Cup containing glass beads to help separation different layer of fecal samples recognized by denaturation to be genetic acceptable for DNA isolation with adding different solution that increase density and capacity. For all samples put in centrifuge to be separated into different layers with adding specific buffer for antigen reaction.

Molecular Analyses

Primers and genetic AC, UG specific for each sampling can be identified and used for detection and separation RNA [25]. Denaturation, protein separation for genetic variation and aliquots mixed buffer [28-30]. All reaction and metabolic assay within same genetic sequences detected at different sequences (ITS-2: 169 bp) *(5′-GTATACATTAA ATAGTGTC CCCC ATTCTAG-, GCAAAT ATCATTAGATT TGATT CTTCCG- -TGGATTT ATTCT CACTACTTA ATTGTTTC GCGAC-* [30].

Conventional PCR and Sequencing

Detection most common isolates commensals with nonpathogenic (i.e., larva and adult worm) but small strongyle, and were special specific nematodes causing clinical pathognomic symptoms (i.e.,) qPCR is method used for quantifying DNA depend on PCR done instructed manually [22-24] and two primers design to matches sequences within templates/probe. There are two chemistry reactions PCR tracks target concentration one of them called coloring waves assay released from these with was5 wt length. The final mix contained 4 µl so Fast Eva Green super mix (Molecular Genetic, RC, USA), first reaction 110°C for 1min followed by 35 reeling amplification at 100°C and annealing for 0.1min., denaturation, analysis 85°C 60sec, 65°C for 60sec, elevation average of temperature for sequencing and annealing stages at .33°C at 44 degree then run same cycling twice for fecal analysis.

Statistical Analyses

McNamara method used for integration cultural of fecal samples so that all data analyzed uses SAS computer test with entering to clinic system for spreading sheet to correlated data about regression coefficient.

Results

Sample Origin

Results of fecal samples counting egg for strongylus was 84 out of the 110 original animal, (76.33%) showed accurately cultring varies from >30upto 9.554 EPG. Counting egg in fecal samples out of >200 was recorded in 38 samples (23.9%).

Current study counting egg per gram fecal samples for strongyles species. Level of egg counting per gram feces was decreased following treatment by Ivermectin in different group of animals by 97 and 98 %, concurrently. The ivermectin + powder anthelmintic added to food consumption decrease egg in feces, however some cases level of egg infecal samples showed high level at 140 day.

Almost of cases with known 57 animals, 74 were known their age and the gender was known for 95 different groups of horses.

The age of the horses ranged from 1 to 35 years of 12 years. Samples originating from 17 mares (40.9%), 46 geldings (57.9%) and five stallions (1.2%) were examined with an average age. On average for 98 horses, the last anthelmintic treatment had been performed 11 months (range 2–196 months) prior to enrolment into the present study.

Identification Fecal Sample for Different Culture Larva

Identification Larval by culture by specific Stoll's method in 57 out of 110 samples obtained from 110 horses with concurrent routine checkup for different cases. Most cultural of 3321.45 Cyathostominae larvae (L3) found in 88.6% of fecal diagnosis (90% of the farm level). Ranging from 3 and 20.325 larvae in 20gm fecal egg count. The EPG and the L/20 g feces conducted by spareman's method of 0.3.65 ($p = 0.01$). Almost 22fecal culture detected larva was 11 for most larva culture in different level of samples detection.

With maximum ratio of samples that 3 out of 78 horses (1.1%). Level of positively in original samples 3 out 62 samples (42.1%) was optimizing generated larvae of strongylus vulgaris in horses.

Prevalence various larva level s in positive samples used for differentiation between nematodes species *Triodontophorus* for free living sheath covered larvae and adult worms.

Real-Time PCR

The real-time PCR was positive in 13 of 89 investigated samples (14.6%; Table 2).

Horse ID	RTE	Stoll's methods	Baerman's techniques	Isolated larvae	p
21	20	+	16	3	27.6
144[a]	20	+	5	4	24.9
235	1040	+++	---	---	33.5
318	100	+	533	0	26.9
334	900	+++	1256	0	35.8
355	---	++	---	---	27.7
375	40	+	28	0	36

412	1720	+++	103	0	23.4
448[b]	20	+	34	0	37
451[c]	20	+	57	0	35.1
460	200	++	535	0	35.8
497	480	+++	1111	3	29.9
520	680	+++	3028	1	35

Table 2: Comparison of genetic detection with larvae culture techniques.

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With regard to horses count of 110 horses, the abundance of eggs was significantly increased in horses with larvae infestation (log DNA median [range]: 7 [5.8–7.5]) compared to healthy dogs (log DNA median [range]: 4.6 [3–6.1]; p = 0.0088). Genetic variation of sequences was also significantly increased in the subgroup

Routine Diagnosis for Samples

Routine check for diagnosis fecal samples was done primary in 4 horses that previously positive for Strongylus vulgaris in follow-up diagnosis. Checkup showed two positive results daily. Case No. 1 (Table 1) showed positive results in first checkup examination (Table 2) further test for confirmed positive 2 months later then further checkup 4 months later first diagnosis to make sure positivity results (Table 1). In this context of the latter two methods fecal samples were taken and analyzed/ for *S. vulgaris* on 11 consecutive days. After primary diagnosis for detection *S. vulgaris* in the fecal samples collected on day 1 and 4 (Table 2). With routine diagnosis and checkup for all horses which diagnosed as negative for *S. vulgaris.* However, due to limited number of further examination horses made statistical analysis hard to be performed.

Comparison of Real-Time PCR and Larval Culture

Alternatives used molecular biology technique with larval culture was evaluated for 85 samples upgrades level. The results / differed significantly comparison between / same results of stoll;s method with p value .02 (Table 3). This is in line with statically analysis ways of computer based analysis / with degree.0 245 with acceptable mild changes.

	Rt PCR –	Rt PCR +	Total
Negative fecal culture	110	5	115
Positive fecal culture +	2	6	8
Total	112	11	123

Table 3: Variation of different parameters categories between RT-PCR and culture of Larva.

Discussion

Experimentally, we found that *Strongylus species* was diagnosed in six of 78 examined horses (3.4%) with fecal culture in 10 of 110 tested horses (1.9%) by real-time PCR. This low occurrence of *S. vulgaris* is in line with previously conducted studies investigating fecal samples from American horses obtained by larval culture with a results ranging from 0.2 to 1.3% [10,19-21]. A comparable prevalence has also been reported in Switzerland [33-42].

Opposite side from old studies in different countries estimated that level of cultural larvae with high compared same as *S. vulgaris* detected by PCR. In a study from Denmark, Nielsen, et al. [6] tested 6 horses from two farms through larval culture and real-time PCR with a result of 11 cases / *S. vulgaris* +ve horses 17.7%. In a number of Poland horses, nematodes were isolated from the intestine and differentiated by necropsy revealing a ratio of 22.8% (16/95) for *S. vulgaris* [43]. An even higher prevalence of 41.3% (19/46) was detected in horses from Europe, via larval culture [44-46]. In differentiating with data from USA Bracken, et al. [47] found a higher rated infection with *S. vulgaris* in Danish horses not only on level with 13.6% (45/71) but on the farm level with 72.2% (13/18). A result of the farm level was created by Nielsen et al. with a ratio of 64.3% (27/42) recorded by larval culture [18].

In the current research work, *S. vulgaris* was found in ten out of 91 involved farms (10.9%). A little presence of *S. vulgaris* in farms from America was further demonstrated by the fact that both the real-time PCR and the larval culture cleared a single *S. vulgaris* +ve horse per farm, solely. These findings are in agreement with another recent USA

prevalence survey, which reported a farm level of 1.04% (2/192) and detected only a single *S. vulgaris* +ve horse per farm with larval media [21].

The concentrated anthelmintic treatment system of the last ten years as well as the long predating period of six to seven months responsible for the current low presence of *S. vulgaris* in USA [3,46,47]. Based on these arguments, a low occurrence for *S. vulgaris* under 5% was reported by Hertzberg, et al. [4] for horses living in Europe. Despite the intensive anthelmintic treatment regime of the last decades, the existence of *S. vulgaris* persists on a low level. A possible reason for this persistence could be a non-complete larvicidal efficacy of ivermectin, as reported by Nielsen, et al. [6] due to his study data in 2014 [48].

A lot of evolution was released that Cyathostominae larvae was in large number than *S. vulgaris* larvae in different fecal cultural samples [21,23,29,48,49]. For example, Ogbourne, et al. [25] refer to a high differ of Strongylus species in equine faecal samples comprising larvae of *S. vulgaris* in less than 10%. Bellaw, et al. [31] said that nearly 1.0% of found larvae were L3 of *S. vulgaris* (86 *S. vulgaris* vs., 72 Cyathostominae larvae).

In today's study, 0.16% of all larvae in 13 *S. vulgaris*-positive samples were L3 of *S. vulgaris* (11 *S. vulgaris* larvae vs. 67Cyathostominae larvae). Both the marginal number of counted *S. vulgaris*-larvae in the larval culture and the occurrence of only one *S. vulgaris* +ve horse/farm might be explained by a low infection rate and by a low shedding of *S. vulgaris* eggs of sick horses.

So that, false negative results might present in samples introduce a low count of *S. vulgaris* eggs. Thus, the dependence on the presence of eggs in the collected fecal sample is the most disadvantage of the treatment of *S. vulgaris* with carpological methods like real-time PCR and larval culture [31,32,50]. Moreover, the relatively long preparation period as same as the dependence of the development of the infected L3 on environmental changes for the seasonality of *S. vulgaris* which may lead to false negative results at certain times of the year [23,51]. Various studies were able to proof a seasonal fluctuation of egg shedding of *S. vulgaris*, with in summer is responsible and a depression in winter [52-55]. Yet, the study at hand was not able to confirm such an influence of seasonality on the detection of *S. vulgaris*-eggs due to a non-seasonal collection of samples and a low number of positive samples. In addition, the current study shows a detection ratio of *S. vulgaris* using real-time PCR in comparison to standard larval culture method [56-58].

A molecular way for the discovery of *Strongylus* spp. was first reported by Campbell, et al. [35]. The specificity of the conventional PCR investigating the ITS-2 gene of *S. vulgaris* has already been analyzed by detecting interspecific variations in the sequence of the ITS-2 *S. vulgaris*, *S. edentatus* and *S. equinus* via conventional PCR and subsequent sequencing [33]. The specificity of the real-time PCR was confirmed by Nielsen, et al. [38] via cross reaction testing between *S. vulgaris*, *S. edentatus, S.* gene between *equinus* and mixture larvae. Therefore, a possible cross reaction with DNA of other equine strongylid nematodes was not expected. Besides the specificity, the sensitivity of the real-time PCR has also been analyzed by Nielsen et al. resulting in of different Cyathostominae a detection limit of a 0.5 Strongylus egg-count [30].

There are a lot disadvantages with fecal cultural takes long time for results for different stages of larva ranged from 10-15 days may be more or less depends species of larvae nematodes [29] though cost-low method might occur due/ for the detection of *S. vulgaris*. A sensitivity of 73% and a specificity of 84% founded by larval culture compared data have been reported by Nielsen, et al. [38]. However, this method is also dependent on the presence of *S. vulgaris* eggs in the investigated samples just like the real-time PCR. Furthermore, the larval culture has the additional disadvantage to necropsy, that false negative results to an inhibited development of L3 which might be caused by fluctuations of temperature, humidity, fungal growth and contamination with free-living nematodes. In this study, the good cultivation of Cyathostominae larvae was showed by the nice correlation among the ―number of Cyathostominae larvae‖ and, the ―FEC‖ of the faecal sample. Cyathostominae larvae were successfully cultivated in 98% of for an inhibited development of larvae might be an time- False negative results due to the latter aspects might experience personnel, since easily occur especially since *S. vulgaris*-positive samples often comprise accidental partial freezing of samples during transportation which was reported for 12 FEC-positive samples which revealed only a limited number of larvae in the larval culture. The negative impact of low d Duncan, et al. [55], Hasslinger [58] and consuming [28], Enigk, et al. [53]. Furthermore, procedure following of supernatant, purification, sedimentation and pipetting might also lead to a loss of larvae. The morphological differentiation of larvae via microscopic examination needs to be conducted detection of *S. vulgaris* larvae is procedure with the design to investigate 100–200 larvae to save time might also contribute to false negative results.

To maintain or even reduce the low by qualified and the occurrence of *S. vulgaris* in Germany and to prevent the introduction of *S. vulgaris* within and among difficult and only a limited number of *S. vulgaris* larvae. The usage cultivation such as steps of the discharging of an aliquot farms, an appropriate optimization of the diagnostic and

management procedures is crucial [3].

Application of real-time PCR for finding of *S. vulgaris* as a routine method could be done in any laboratory with appropriate preparation for the real-time PCR-method [45]. Strongylus eggs had been recovered by sedimentation/ flotation method can directly be used for the DNA-extraction and subsequent real-time PCR. In now study, a FEC of 20 EPG was enough for approve of *S. vulgaris* by real-time PCR.

Since the follow-up visits in this study showed that not every single reproduces an initially *S. vulgaris* +ve result, the determination of used faecal samples per horse by real-time PCR for an adherent detection of *S. vulgaris* might be useful in the daily routine diagnostics. Moreover, all horses of a herd or at least ―high risk patients‖ like newcomers and horses with unknown history should be diagnosed one by one [3]. Preliminary to an integration into a new place, thorough test for *S. vulgaris* is essential too for horses originating from countries with a high prevalence for *S. vulgaris* like England, a part of Germany and Spain [36,43,44].

Conclusion

Current studies provide comparative results for diagnosis S. vulgaris by advanced molecular biology and convential fecal culture method both suggested improvement for diagnostic method for *S. vulgaris* in fecal samples with high positive rate by the real-time PCR is recent tool for diagnostic protocol and prevention with control parasitic infestation for early diagnosis by genetic marker with no confusion for samples examination.

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