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Benzimidazole resistance in equine cyathostomins in India

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ABSTRACT

Benzimidazole resistance is a major hindrance to the control of equine cyathostominosis throughout the world. There is a paucity of knowledge on the level of benzimidazole resistance in small strongyles of horses in India. In the present study, allele-specific PCR (AS-PCR) that detects F200Y mutation of the isotype 1 β-tubulin gene and faecal egg count reduction test (FECRT) were used for detecting benzimidazole resistance in equine cyathostomin populations in different agro-climatic zones of Uttar Pradesh, India. Results of the FECRT revealed prevalence of benzimidazole resistance in cyathostomins in an intensively managed equine farm in the mid-western plain (FECR = 27.5%, LCI = 0) and in working horses (extensively managed) at three locations in central plains of Uttar Pradesh (FECR = 75.7–83.6%, LCI = 29–57%). Post-treatment larval cultures revealed the presence of exclusively cyathostomin larvae. Genotyping of cyathostomin larvae by AS-PCR revealed that the frequency of homozygous resistant (rr) individuals and the resistant allele frequency was significantly higher ($p < 0.001$) in the intensively managed farm in the mid-western plain and in working horses at two locations in central plains of the state. The resistant allele (r) frequency in cyathostomins was significantly higher ($p < 0.05$) in Vindhyan and Tarai and Bhabar zones of Uttar Pradesh. The prevalence of benzimidazole resistant allele (*r*) was significantly higher ($p < 0.05$) in cyathostomins of intensively managed horses (allelic frequency—0.35) as compared to extensively managed horses (allelic frequency—0.22). The widespread prevalence of benzimidazole resistant alleles in equine cyathostomins in Uttar Pradesh, India, necessitates immediate replacement of the drugs of benzimidazole group with other unrelated effective anthelmintics for management and control of equine cyathostomins.

1. Introduction

Equine strongylosis, caused by large strongyles (sub-family Strongylinae) and small strongyles (sub-family Cyathostominae), is ubiquitous in grazing horses throughout the world. Virtually every horse can be expected to be infected with these parasites, which often account for a parasitic load up to 100% in grazing horses (Kaplan et al., 2004). Cyathostomins are usually considered as less pathogenic as compared to large strongyles, especially *Strongylus vulgaris*, but they are the major cause of health problems in equines (Herd, 1990) as mass emergence of encysted larvae of small strongyles is known to cause larval cyathostominosis, which has a case fatality rate of around 50% (Love et al., 1999). In India, equine strongylosis is currently controlled by chemotherapy with three groups of broad spectrum anthelmintics *viz*., benzimidazoles, tetrahydropyrimidines and macrocyclic lactones. Of these, benzimidazoles are in use for more than the last four decades (Yadav et al., 1984; Bagherwal et al., 1989; Banerjee et al., 2002; Singh et al., 2002a,b; Khajuria et al., 2006; Sharma et al., 2011a; Singh et al., 2012). However, frequent treatment, under-dosing, high stocking rates, high pasture contamination with infective larvae, shorter prepatent period of these parasites and shrinkage in grazing lands due to massive industrialization have contributed to rapid emergence of benzimidazole resistant strains of gastrointestinal nematodes in India (Yadav and Garg, 2005). Benzimidazole resistance in cyathostomes was first reported by Drudge and Lyons (1965). Since then, benzimidazole resistant cyathostomes have been recorded around the world (Peregrine et al., 2014). Apart from two reports of benzimidazole resistance in cyathostomes from India (Pal, 2002; Kumar and Vatsya, 2014), there are no reports from Asia.

The commonly used methods for detection of benzimidazole resistance in equine strongyles include, faecal egg count reduction test (FECRT, *in vivo*), egg hatch assay (EHA, *in vitro*) and micro agar larval development test (MALDT, *in vitro*). A common limitation of these tests is their relatively low sensitivity, as they detect resistance only when more than 25% of the worm population is resistant to benzimidazoles (Martin et al., 1989). Recently,

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the molecular mechanism that confers benzimidazole resistance in ruminant trichostrongylids as well as in equine cyathostomins involving a phenylalanine (TTC) to tyrosine (TAC) mutation at codon 200 of the isotype 1 β-tubulin gene (F200Y) has been identified (Kwa et al., 1994; Elard et al., 1996; Pape et al., 1999, 2003; von Samson-Himmelstjerna et al., 2001, 2002b). A similar mutation at codon 167 (F167Y) is also involved in BZ resistance in nematodes including cyathostomes (Prichard, 2001; Pape et al., 2003). This F167Y mutation may be found in the absence of F200Y mutation, but has been observed to occur rarely in the field (Silvestre and Carbaret, 2002; Hodjkinson et al., 2008). However, F167Y mutation has never been reported from either ruminant trichostrongylids or equine cyathostomins from India. This knowledge of the molecular basis of benzimidazole resistance has allowed development of molecular tools for early and rapid detection of benzimidazole resistance in equine cyathostomins *viz.* allele-specific PCR (AS-PCR) and real time PCR for detecting F200Y polymorphisms (von Samson-Himmelstjerna et al., 2002a, 2003), and pyrosequencing assays for detecting F167Y polymorphism (Lake et al., 2009). The greatest advantage of these tools is that the emergence of a first mutant individual can be detected in a worm population (Elard et al., 1998).

Keeping in mind the potential threat posed by widespread emergence of benzimidazole resistance in small strongyles of equines throughout the world, and paucity of information on the level of benzimidazole resistance in equine strongyles in India, the present investigation was planned to investigate the status of benzimidazole resistance in cyathostomins of horses reared under different management systems in different agro climatic zones of Uttar Pradesh, India using FECRT and AS-PCR that detects F200Y mutation.

2. Materials and method

2.1. Study area

The study area, Uttar Pradesh, is a north Indian state located between 23°52′N and 31°28′N latitudes and 77°3′E and 84°39′E longitudes. According to the 19th Livestock Census of India, Uttar Pradesh has the largest population of equids (DAHDF, 2014). The state has a humid subtropical climate with average temperatures varying from 8.5–21.9 °C in winter (December and January) to 25.5–45.0 °C in summer (May and June). Rainfall in the area ranges from 600 to 2000 mm from July to September with an average of 1072 mm. The humidity reaches more than 90% during the rainy season (July and August). The study on the prevalence of benzimidazole resistance was carried out on horses reared under intensive management system at government/private farms where the horses are raised in a confined area with a defined pasture and regularly dewormed with anthelmintics (3–4 times/year) as well as on working horses reared under extensive management system (nomadic herding) where anthelmintics are administered to the horses occasionally as detailed in Table 1 using FECRT and/or AS-PCR.

2.2. Faecal egg count reduction test

FECR tests were carried out on horses during April–October, 2014 at 9 different locations as described in Table 1. Faecal samples were collected directly from the rectum of individual adult horses (4–8 years of age), in properly labelled polythene bags and transported on ice to the laboratory for estimation of eggs per gram of faeces (epg) by a modified McMaster technique with a sensitivity of 15 epg (MAFF, 1986). While collecting the faecal samples from horses, the history and frequency of anthelmintic use on the farm was also recorded. The horses having an epg of more than 150 were selected for FECRT. The weight of a horse was estimated according to the method described by Carroll and Huntington (1988). On day zero, horses were treated with fenbendazole (Panacur, Intervet, India Pvt., Ltd., Pune) at the dose rate of 10 mg/kg body weight orally. Rectal faecal samples were again collected 14 days post-treatment (14 DPT) from treated horses and again epg was estimated. The arithmetic mean, percent reduction and lower 95% confidence interval (LCI) were calculated as described by Coles et al. (1992, 2006). Benzimidazole resistance was considered present if (a) the percent FECR was less than 90% and (b) the LCI was less than 80%. If only one of the two criteria was met, the resistance was suspected (Lester et al., 2013).

Pooled faecal samples of horses from different locations were cultured on day 0 and 14 DPT for collection of third stage strongyle larvae and subsequent species-specific identification (MAFF, 1986).

2.3. Allele specific PCR

AS-PCR was carried out on genomic DNA isolated from equine cyathostomin larvae harvested from faecal samples of horses at 9 different locations (Table 1) as per the method described by von Samson-Himmelstjerna et al. (2002a) and Coles et al. (2006). Genomic DNA was isolated from one hundred individual exsheathed larvae of small strongyles from each location. Briefly, 4 ml of pooled larval suspension from each location (600 larvae/ml of water) was incubated with 180 μl sodium hypochlorite (aqueous solution, about 3.5% active chlorine) for 10 min at room temperature. Individual larva in 2 μl water was pipetted under the microscope into a PCR tube and incubated at 41 °C overnight with 6 μl of extraction buffer (1 mM Tris–HCl, 0.1 mM EDTA, 5 mg/ml proteinase-K). Following incubation, inactivation of proteinase-K was done at 95 °C for 20 min. The tubes containing the digested larval suspension were then stored at −20 °C till used as templates for PCR amplification.

A total of 100 infective cyathostomin larvae, harvested from pooled faecal samples of horses from each location, were individually genotyped as homozygous susceptible (SS), homozygous resistant (rr) and heterozygous (rS) by allele specific PCR that detects F200Y mutation in isotype 1 β-tubulin gene. For each larvae, AS-PCR was performed in 2 separate PCR tubes, each containing either of the 2 forward primers i.e. susceptible allele primer (CN24FS- ggttgaaaatacagacgagacttt) or resistant allele primer (CN25FR- ggttgaaaatacagacgagactta) and a common reverse primer (CN30R- agcagagaggggagcaaagccagg). Preparation of the reaction mixture and the reaction conditions of PCR were similar to those described by Coles et al. (2006). The amplicons were vistualized by 2.0% agarose gel electrophoresis.

2.4. Statistical analysis

The results of genotyping of cyathostomin larvae by AS-PCR from each location were analysed by Chi-square test using SPSS software version 16. Spearman rank correlation coefficient (r_s) and p value between FECR% and pre-treatment susceptible allele percentage at 7 locations where both FECRT and AS-PCR were performed (Table 1) were determined using Microsoft Excel 2007 software.

3. Result

Results of FECRT and AS-PCR indicated widespread prevalence of benzimidazole resistant equine cyathostomins in Uttar Pradesh, India (Table 1). Out of the 9 locations where FECRT was performed, benzimidazole resistance in cyathostomins was detected at 4 locations (44.4%) *i.e.*, at an intensively managed equine farm in