The Pharma Innovation Journal 2019; 8(6): 1092-1097

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(6): 1092-1097

www.thepharmajournal.com
Received: 16-04-2019
Accepted: 18-05-2019

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Detection and quantification of *Oxalobacter formiginase* in soybean crop residue fed cattle

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Abstract
Oxalate rich plants may become important forage resources for ruminants when other feedstuffs are in short supply. *Oxalobacter formigenes* is considered as the main oxalate-degrading bacteria in ruminants. The identification and quantification of these bacteria is essential in animals fed with oxalate rich plants. The faecal samples from the animals fed on soybean crop residue and healthy control animals which were not fed on soybean crop residue were collected in a sterile bottle and subjected for their molecular analysis for identification and relative quantification of *Oxalobacter formigenes.* The genomic DNA was extracted from capsule containing *Oxalobacter formigenes* 700 million by using PowerFecal DNA isolation kit (MoBio Laboratories, Inc.) for standardization of PCR. The DNAs' from collected faecal samples of animals fed on SCR were extracted as per the experimental protocol supplied by MoBio Laboratories with slight modifications as per the necessity. Out of 26 faecal samples of SCR fed animals, 15 (57.69%) were positive and 11 (42.31%) were negative for *Oxalobacter formigenes* while out of 12 control samples (SCR not fed) 3 samples (25.00%) were positive and 9 (75.00%) were negative for *Oxalobacter formigenes.* Overall 18 faecal samples (47.37%) were expressed positivity for *Oxalobacter formigenes.* The results for expression of frequency of *Oxalobacter formigenes* revealed higher frequency of *oxc* gene in the faecal samples of animals fed with SCR than that of healthy control animals. Relative quantification of *O. formigenes* revealed high Ct value (28.45 and 29.52)in oxalate toxicityand SCR not fed animals (ranged from 29.16 to 33.28) than SCR fed (ranged from 20.85 to 27.10) and positive control (18.78) indicated more quantity of *O. formigenes* in the faecal samples of animals fed with SCR than SCR not fed and animals with oxalate toxicity.

Keywords: *Oxalobacter formigines,* soybean crop residue, cattle

1. Introduction
Feed scarcity is the primary constraints for livestock keepers in addition to the livestock health. Having only 4% of total cropping area under fodder cultivation, the country faces a net deficit of 61.1% green fodder, 21.9% dry crop residues and 64% concentrate feeds (Datta, 2013) [2]. Crop residue is the most important single fodder resource providing about 44% of the overall feed resources (NIANP 2003) which support low level of production (Wanapat *et al.*, 2009) [25]. Further the conventional sources of feed, both roughages and concentrate are not enough to meet requirement of the country (Ranjhan, 2003) [19]. As a result, the bulk of feed available for ruminants in these situations are the crop residues. The crop residues have low nutritional value and are bulky and fibrous in nature. However, these feed resources are not well managed, especially where these are available in plenty (FAO**,** 2012) [3].
Majority of livestock keepers are feeding crop residues like sugarcane top, wheat and paddy straw and now in the states like Maharashtra where recent cropping pattern has been changed and producing more soybean crop, are feeding soybean crop residue to the animals. Production of soybean in India is dominated by Maharashtra and Madhya Pradesh which contributes 89% of the total production (FICCI Report, 2015). SOPA (2014) along with other associate agencies conducted extensive Crop Survey in three major Soybean Producing States of India as Madhya Pradesh, Maharashtra and Rajasthan. Ramachandra *et al.* (2007) [18] predicted that crop residue's contribution to the Indian feed budget will reach almost 70% by the year 2020.
A wide range of plants including food stuff, *Chenopodium album* (bathu), *Spinacea oleracea* (spinach) and forages, *Cenchrus ciliaris* (buffel grass), *Pennisetum clandestinum* (kikuyu grass), *Pennisetum purpureum* (napier grass), Rice straw, *Digitaria decumbens* (pangola grass), *Amaranthus spp.* (pigweed), *Rheum rhaponticum* (rhubarb), *Salsola kali* (Russian thistle), *Setaria sphacelata* (setaria) and *Beta vulgaris* (sugar beets) contain large amounts of

oxalate which in some circumstances may lead to toxicity in farm animals. In India, *Pennisetum glaucum* (bajra), *Trifolium alexandrinum* (barseem), *Sorghum bicolor* (chari), *Megathyrsus maximus* (guinae grass), *Zea mays* (maize), *Brassica campestris* (mustard), *Napier Bajra Hybrid* (NBH) and *Avena sativa* (oats) are the commonly used forages for dairy animals. Napier grass represents a major feed source for ruminants in tropical and subtropical areas that also contains up to 3.8% soluble oxalate (Rahman *et al*., 2006; Rahman *et al*., 2010) [16, 17]. Five outbreaks of NBH toxicity in farm animals associated with excessive accumulation of oxalate occurred in Punjab during the year 2009-2010. The rumen microbial population has the first opportunity to digest any feed consumed by the ruminant and anything that affects the rumen ecosystem will ultimately affect what and how nutrients are available to the animal for productive purposes. When the rumen becomes dysfunctional, feed digestion is impaired and animal become susceptible to a range of metabolic and digestive disorders. Digestive disorders in cattle directly affect the economics of dairy farm due to impact on production. In general, oxalate content is highest in the leaves, then in the seeds, and lowest in the stems of soybean (Singh *et al*., 1972) [23]. Oxalate, consumed in sufficient quantity, may bind with calcium (Ca) in the intestines and the blood to form insoluble Ca oxalate, which may lead to low serum Ca levels and renal failure (James, 1972) [7]. Increased absorption of oxalate from the intestinal tract is due to the deficiency or complete absence of intestinal oxalate degrading bacteria, predominantly *Oxalobacter formigenes*. Normally these bacteria would degrade approximately 50 – 80 % of the dietary oxalate into carbon dioxide and formate, but in their absence or diminished number, more oxalate is available for absorption and consequently more oxalate is being excreted through urine (Hoppe *et al.*, 2005) [6].

Oxalobacter formigenes, are inhabitants of the rumen and also of the large bowel of man and other animals where their actions in destruction of oxalic acid may be of considerable importance to the host. Many workers have studied, particularly in humans, the importance of *Oxalobacter formigenes* in the degradation of oxalic acid in the gut and its role in the occurrence of urinary stones/urolithiasis or renal failure. However, such study was not yet been carried out in the ruminants, therefore the present investigation regarding molecular analysis of faecal samples of cattle fed on soybean crop residue for identification and relative quantification of *Oxalobacter formigenes*, oxalate degrading bacteria was carried out.

2. Materials and Methods

2.1 DNA Isolation

The genomic DNA was extracted from capsule containing *Oxalobacter formigenes* 700 million by using Power Fecal DNA isolation kit (MoBio Laboratories, Inc.) for standardization of PCR. The DNAs' from collected faecal samples of animals fed on SCR were extracted as per the experimental protocol supplied by MoBio Laboratories with slight modifications as per the necessity. The each sample was homogenized in a 2 ml bead beating tube containing garnet beads. Cell lysis of host cells as well as microbial cells was facilitated by both mechanical collisions between beads and chemical disruption of cell membranes, ensuring efficient extraction from even the toughest of microorganisms. Then inhibitor removal technology (IRT) was used to remove common substances in samples that interfere with PCR. Total genomic DNA was captured on a silica spin column. DNA was then washed and eluted for further PCR analysis including qPCR for identification and quantification of *Oxalobacter formigenes*, oxalate degrading rumen bacteria.

2.2 Polymerase chain reaction (PCR)

Oxalobacter formigenes specific oxc gene was amplified by using primers depicted in Table 1. The PCR was carried out on 2.0 µl of DNA in gradient mastercycler (Eppendorf, USA) as per the reaction components and cycling conditions depicted in Table 2 and 3.

Table 1: Details of primers used in the present study.

Gene	Primer sequences (5'->3')	Product size	Reference
Oxc	F- ATGTAGAGTTGACTGATGGC R- AAAACGCTGACCGTCATCTT	500bp	Jiang *et al*., 2011

Table 2: PCR reaction component of *oxc* gene

Component	Quantity (µl)
PCR mastermix 2x	12.50
Forward primer	1.25
Reverse primer	1.25
Template DNA	2.00
Nuclease free water	7.8
Taq polymerase	0.2
Total	25.00 µl

Table 3: The cycling conditions in master gradient cycler for *oxc* gene

Step-I	Initial denaturation	94ºC for 7 min	1 cycle
Step-II	Denaturation	94ºC for 30 sec	30 cycles
	Annealing	48ºC for 40 sec	
	Extension	72ºC for 60 sec	
Step-III	Final extension	72ºC for 10 min	1 cycle
Step-IV	Hold	4ºC	

2.3 Confirmation of PCR product

The PCR product was loaded in agarose gel (1% agarose in 0.5 x trisborate EDTA buffer, ethidium bromide (0.5 µg/ml) along with standard molecular size marker (100 bp DNA ladder). The gel was electrophoresed (Horizontal gel electrophoresis system, Genaxy). Amplified products were separated on agarose gel and observed by ultraviolet transilluminator and photographed in a gel documentation system (Gel-Pro Analyzer, Syngene, USA).

2.4 Quantification of *Oxalobacter formigenes* by qPCR

The relative quantification of *Oxalobacter formigenes* in faecal samples was done using real-time PCR method described by Jaing *et al.* (2011) to see *oxc* gene expression in SCR fed and SCR not fed animals. The real-time PCR primers used were5'ATGTAGAGTTGACTGATGGC3' as the sense primer and 5' AAAACGCTGACCGTCATCTT 3' as the antisense primer based on sequence alignments. The real-time PCR reaction was done in triplicate using AB Applied Biosystems SYBR Green PCR Kit, 1.25 µM of each primer and 100 ng faecal DNA. The assay was performed in a 96-well optical reaction plate with an optical adhesive film using a Roche LC-96, USA real-time PCR apparatus. The PCR reaction was done with incubation at 95ºC for 15 minutes to activate HotStarTaq DNA polymerase and 45 following cycles, including 94ºC for 30 seconds, 53 - 60ºC for

30 seconds and 72°C for 1 minute.

3. Results and Discussion

3.1 Identification of *Oxalobacter formigenes*

Overall 26 faecal samples from the cattle fed with soybean crop residue and 12 faecal samples from the healthy control cattle (not fed SCR) were subjected for DNA isolation. Polymerase Chain Reactions were carried out with *Oxalobacter formigenes* specific primer pairs 5′ ATGTAGAGTTGACTGATGGC 3′ and 5′ AAAACGCTGACCGTCATCTT 3′. The results are shown in Table 4 and Fig. 1 to 2.

Table 4: Overall frequency of *oxc* gene of *Oxalobacter formigenes* in faecal samples of SCR fed and healthy control animals

	Frequency of *oxc* gene in SCR fed animals (n=26)		Frequency of *oxc* gene in healthy control animals (n=12)		Total	
	Number	Per cent	Number	Per cent	Number	Per cent
Positive	15	57.69	3	25.00	18	47.37
Negative	11	42.31	9	75.00	20	52.63
Total	26	100.00	12	100.00	38	100.00

Out of 26 faecal samples from animals fed with SCR, 15 (57.69 %) were positive and 11 (42.31 %) samples were negative for *Oxalobacter formigenes* while out of 12 control faecal samples (SCR not fed animals) 3 samples (25.00%) were positive and 9 (75.00%) were negative for *Oxalobacter formigenes.* Overall 18 faecal samples (47.37%) expressed positivity for *Oxalobacter formigenes.* The results for expression of frequency of *Oxalobacter formigenes* revealed higher frequency of *oxc* gene in the faecal samples of animals fed with SCR than healthy control (SCR not fed) animals.

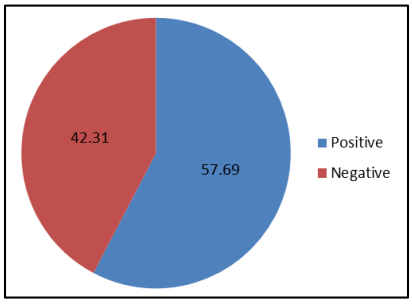

Fig 1: Per cent frequency of *oxc* gene in SCR fed animals

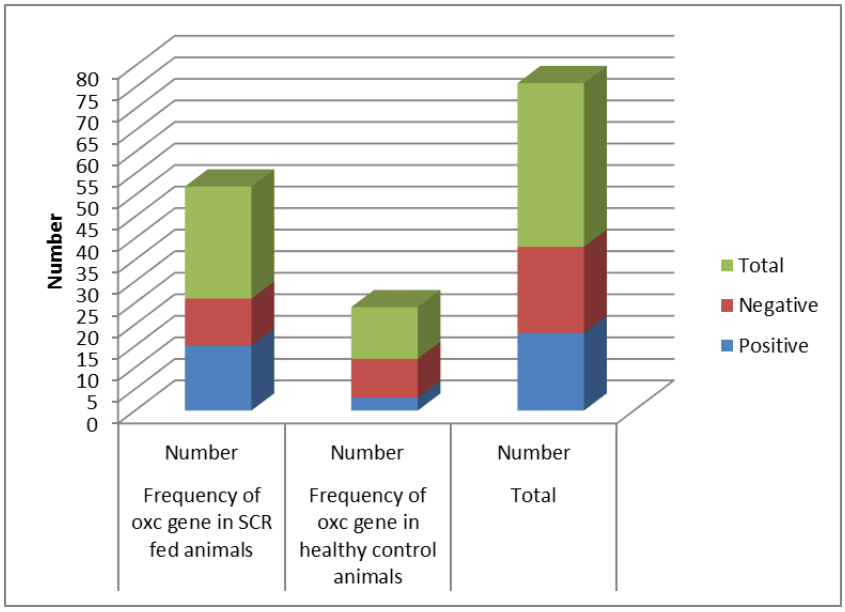

Fig 2: Frequency of *oxc* gene in SCR fed and SCR not fed animals

Oxalobacter formigenes expresses a unique gene required for catabolising oxalate (oxc, encoding oxalyl coenzyme A decarboxylase) which was cloned and sequenced. Sequencing of the 5' ends of *oxc* of *O. formigenes* was identified with unique and highly conserved regions, allowing for synthesis a specific PCR primer pair. The amplification of whole faecal DNA with this specific primer pair provides a rapid diagnostic tool to detect *O. formigenes.*

In the present study *O. formigenes* were detected more in SCR fed cattle than SCR not fed cattle indicated oxalate content in SCR which emphasized role of *O. formigenes* in degradation of oxalate by its more activation. However, this result is in disagreement with findings of Jasim and Abd Al-Abbas (2016) who detected specific *oxc* gene for *Oxalobacter formigenes* in 96 % of faecal samples from healthy comparing with human patients. *O. formigenes* is important in maintaining oxalate homeostasis and its absence from the gut may increase the risk of calcium oxalate urolithiasis (Kwak *et al.*, 2003) [13].

The simplest hypothesis to explain the effect of *O. formigenes* colonization on urinary stone disease is that the bacteria consume oxalate in the colon, decrease the amount of oxalate available for absorption and, consequently, decrease the amount excreted in urine. It may be further hypothesized that the concentration of the soluble oxalate anion in the intestinal lumen is a critical determinant of the amount of oxalate absorbed (Jiang *et al.*, 2011) [9].

Increased absorption of oxalate from the intestinal tract is due to the deficiency or complete absence of intestinal oxalate degrading bacteria. Normally these bacteria would degrade

approximately 50-80% of the dietary oxalate into carbon dioxide and formate, but in their absence or diminished number, more oxalate is available for absorption and consequently more oxalate is being excreted through urine (Hoppe *et al.,* 2005) [6]. *Oxalobacter formigenes* gets its energy only by breakdown of oxalate in colon. *Oxalobacter formigenes* contains two enzymes oxalyl coenzyme decarboxylase and formyl coA transferase. These enzymes degrade oxalate to CO2 and formate which is further metabolized and excreted through faeces (Sidhu *et al.*, 1997 and Rokade *et al*., 2015) [21, 22].

3.2 Quantification of *Oxalobacter formigenes*

The faecal samples from SCR fed and SCR not fed (control) animals which were identified positive for *Oxalobacter formigenes* by PCR, subjected for relative quantification by using real time PCR (qPCR) and the results of the same are depicted in Table 5 and 6 and Fig. 3.

Table 5: Relative quantification of *oxc* gene in SCR fed animals by qPCR

Sr. No.	Sample No.	Ct value
1	03	26.89
2	05	23.42
3	06	24.54
4	07	20.85
5	09	26.81
6	10	25.43
7	12	26.75
8	13	22.14
9	18	27.10
10	19	21.48
11	21	22.64
12	23	24.62
13	24	26.51
14	25 (Clinical)	28.45
15	26 (Clinical)	29.52
16	Positive control	18.78

From the above table, it was revealed that in SCR fed animals Ct value ranged from 20.85 to 27.10 while two animals with oxalate toxicity shown Ct value of 28.45 and 29.52 as compared to Ct value 18.78 of positive control. The Ct value denotes cycling threshold of *oxc* gene indicated amplification (Plate 1 and 2). It has indicated that *Oxalobacter formigenes*, oxalate degrading rumen bacteria, were more in quantity in the faecal samples of animals fed with SCR than SCR not fed and in animals with oxalate toxicity; however, they were less in quantity than positive control. The less quantity of *Oxalobacter formigenes* in the faecal samples of animals with oxalate toxicity might be due to exhaustion of these bacteria to degrade and detoxify the large quantity of oxalic acid in the rumen and this might be one of the reason for occurrence of oxalate toxicity. The present findings substantiate the findings of Han *et al.* (1995) who reported that the count of *Oxalobacter formigenes* was significantly low in patients with urolithiasis due to oxalic acid than that in control and suggested reduction in *O. formigenes* was an important factor in calcium oxalate calculi formation. Kleinschmidt *et al.* (1993) also reported that the density of *O. formigenes* (c.f.u./g) of faeces was inversely correlated with the frequency of episodes of kidney stone formation, noting a complete absence of this bacterium in kidney stone patients having four or more episodes. Furthermore, the absence of *O. formigenes* and a lower rate of oxalate degradation were reported in faecal samples obtained from patients with enteric hyperoxaluria. Prokopovich *et al.* (2007) [15] measured intra-stool and inter-stool sample variability in the amount of *O. formigenes* by real-time PCR that did not correlate with the quantity of oxalate in stool. Most subjects had a faecal colonization of less than 4×10^4 per gm stool. They also showed the relationship of *O. formigenes* number with Ct value.

Table 6: Relative quantification of *oxc* gene in SCR not fed (Control) animals by qPCR

Sr. No.	Sample No.	Ct value
1	3	33.28
2	5	29.16
3	8	31.62
4	Positive Control	18.78

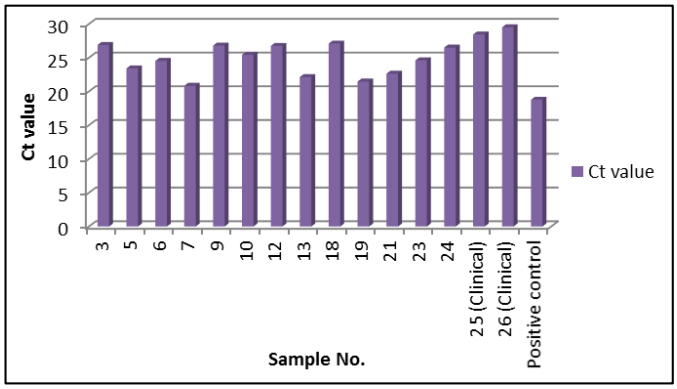

Fig 3: Ct values of positive *oxc* gene in SCR fed animal's faecal samples amplified in qPCR

The three faecal samples positive for *O. formigenes* were quantified with Ct value 33.28, 29.16 and 31.62 and revealed low quantity of *O. formigenes* as compared to positive control (Ct value 18.78) (Fig. 3). Presence of *Oxalobacter formigenes* in these three healthy animals indicated oxalic acid detoxification activity of *O. formigenes* in rumen and preventing oxalate toxicity. Kwak *et al.* (2001) [12] identified *Oxalobacter formigenes* in fresh and frozen faecal samples from healthy and patients with urolithiasis by PCR technique and concluded that *O. formigenes* can be easily and efficiently identified using PCR-based detection system and they found that the colonization rate of *O. formigenes* in patients with urolithiasis was significantly lower than that in healthy volunteers free from urolithiasis. Further, they observed identical results of PCR-based assay in the frozen and fresh faecal samples. Kumar *et al.* (2004) also studied stool samples for *O. formigenes* using PCR in patients with calcium oxalate renal stones and inflammatory bowel disease and revealed patients with calcium oxalate renal stones and inflammatory bowel disease had lower *O. formigenes* than healthy control patients and interpreted that absence of *O. formigenes* colonization as a risk factor in absorptive or enteric hyperoxaluria.

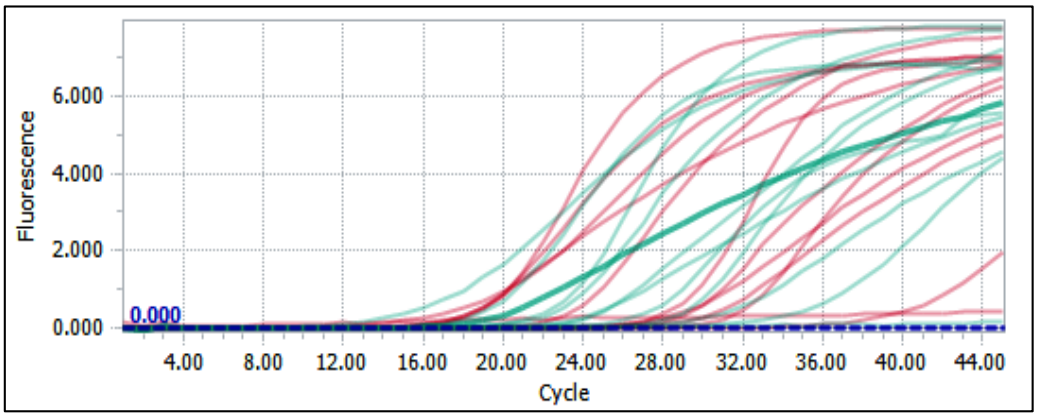

Plate 1: Amplification curve of oxc gene from field faecal samples by qPCR

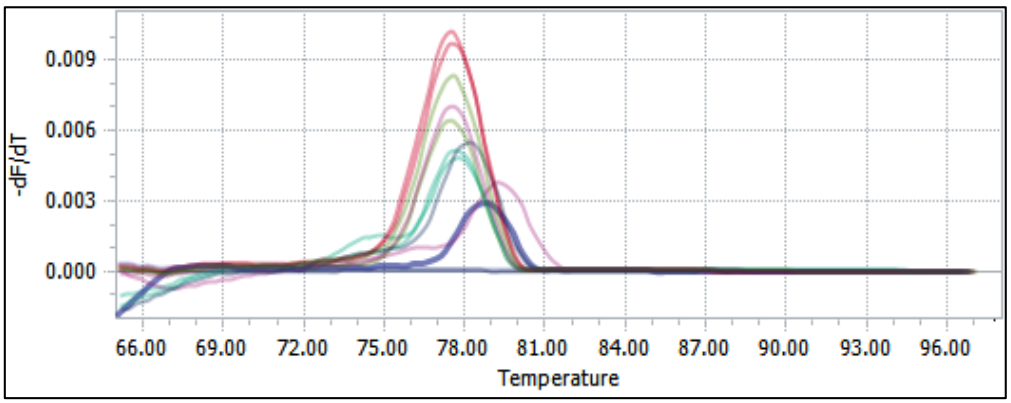

Plate 2: Melting peak of *oxc* gene from field samples

4. Conclusion

It can be concluded that the expression of frequency of *Oxalobacter formigenes* revealed higher frequency of *oxc* gene in the faecal samples of animals fed with SCR than that of healthy control animals. Relative quantification of *O. formigenes* revealed high Ct value (28.45 and 29.52) in oxalate toxicity and SCR not fed animals (ranged from 29.16 to 33.28) than SCR fed (ranged from 20.85 to 27.10) and positive control (18.78) indicated more quantity of *O. formigenes* in the faecal samples of animals fed with SCR than SCR not fed and animals with oxalate toxicity.

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