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Differential Expression Profiling of Myogenic Regulatory Factor Genes in Postnatal *Longissimus dorsi* Muscle of Indigenous and Large White Yorkshire Breeds of Pigs

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ABSTRACT

Pigs form a very important component of the Indian livestock. It has immense potential to ensure nutritional and economic security for the weaker sections of the society. Pork is a nutritious food that is commonly consumed worldwide. The current study was planned to investigate the distinction in the relative expression of myogenic regulatory factors (MRF) genes (*MyoD, Myf5, Myf6, Pax7 and Pax3*) and their role in the postnatal myogenesis *Longissimus dorsi* muscles of indigenous and Large White Yorkshire (LWY) breeds. These genes help in muscle differentiation and regeneration of skeletal muscles. *MyoD* has shown significantly ($P<0.05$) higher quantitative transcript levels in indigenous pigs than in LWY. There is no significant difference in the transcript levels of *Myf5* and *Myf6* have been observed in the indigenous pigs whereas both the genes have shown significantly ($P<0.05$) higher quantitative transcript levels in LWY. Further, no statistically significant ($P<0.05$) difference for *Pax3* and *Pax7* have been recorded for both the breeds. The current study on relative expression of transcript levels of MRFs in LWY and indigenous pigs presents them as candidate genes for body growth rate. The current study will aid in understanding the genetic basis for myogenesis in postnatal stage. Moreover, it may act as steppingstone for the identification of marker genes related to body growth and meat quality in indigenous breeds.

HIGHLIGHTS

- We studied differential expression of myogenic regulatory factor genes in postnatal *Longissimus dorsi* muscle of indigenous and Large White Yorkshire breeds of pigs
- The genes under current study will help in muscle differentiation and regeneration of skeletal muscles.
- The current study will aid in understanding the genetic basis for myogenesis in postnatal stage.

Keywords: Indigenous, *MyoD, Myf5, Myf6, Pax7, Pax3*

Pigs form a very important component of the Indian livestock. Pigs are generally raised by economically weaker section of the society which not only provide them with a better nutritional support but also serves as an important source of livelihood (Sulabh *et al.,* 2017). As a cheap source of healthy animal protein pigs can meet the requirement for the weaker section of the society (Chhabra and Samantaray, 2013). Pig as compared to other livestock species has a great potential to contribute to faster economic return to the farmers due to better-feed conversion efficiency, early maturity and short generation interval. Ten indigenous pig breeds have been recognized in India (Kaur *et al.,* 2020). The larger population consisting

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of an un-descript type of pigs usually have less production ability (Sulabh *et al.,* 2017). However, implementation of proper cross-breeding programmes development of popular and locally acceptable new breeds by crossing indigenous pigs with high yielding exotic breeds have resulted in an expansion in the popularity of pig rearing. Acceptance of the pig meat by the society has resulted in an increase in the demand.

In Punjab there are more than 200 piggery farmers having more than 30,000 pigs. According to GIO there is 0.03 million pigs population. *Indigenous breeds of pig have poor growth rate, small litter size but the quality of pork is high.* Pork from indigenous breed is tender and juicy (Sodhi *et al.,* 2014). *LWY has* higher growth rate, better feed efficiency and high prolificacy rate. Muscularity and growth rate are among the prime economic traits for meat animals. Clear evidences have established that size of muscle fiber; number of fibers; fiber area and density of fibers are in close vicinity with the traits related to meat quality (Hwang *et al.,* 2010).

Muscle regulatory factors (MRFs), are components of basic helix- loop- helix (bHLH) family and are involved in the myogenesis. Myogenesis and transcription of the genes specific for muscles is controlled by bHLH transcription factors (te Pas *et al*., 2007). Myogenic determination factor (*MyoD*), myogenic factor-6 (*Myf6*) and myogenic factor-5 (*Myf5*) are the multifaceted members of MRF. *MyoD* is implicated in the differentiation of skeletal muscles. It has been reported that *MyoD* and paired box transcription factor-7 (*Pax7*) co-express in the actuated satellite cells (Xu *et al.*, 2018). *Pax7* induces self-regeneration of satellite cells and can be one of the candidate genes to affect the feisty stages of post-natal muscle growth in the pig (Patruno *et al*., 2007). *Myf6* has been recognized for encrypting bHLH transcription factors and significantly affect the differentiation of muscle fibers (Wyszynska-Koko and Kuryl, 2004). Further, it has tendency to express more in the adult skeletal muscle tissue of lean breeds (te Pas *et al*., 2000). Earlier reports suggested that anomaly in the cleavage of MRFs can have deep effects on the growth of skeletal muscles (Allen *et al*., 2010). Although the relationship between the weight of carcass, characteristics of muscle fiber and traits related with pork quality is not fully understood, but still muscle mass and the weight of the pigs are related to fiber composition (Yang and Kim, 2006).

Meager reports are available on comparative transcriptomic studies on indigenous breeds of pigs. Therefore, the relative analysis of transcript levels of MRFs in the postnatal myogenesis of *longissimus dorsi* muscles in indigenous pigs and LWY breeds have been targeted in the current study, so that these can be pondered as candidate genes for muscle growth during the breed improvement programs.

MATERIALS AND METHODS

The current study was carried out at (Department of Animal Biotechnology and College of Animal Biotechnology) Guru Angad Dev Veterinary and Animal Sciences University (Ludhiana).

Collection of tissues

In the current study, six each pure breed adult animals each from Indigenous and LWY breeds were chosen. The *longissimus dorsi* muscle tissue samples between 12th and 13th rib spaces were harvested instantly following the slaughter from slaughterhouse. Tissue samples were transported immediately from slaughterhouse to lab in RNA later solution and dry ice. Later, the samples were later kept at -80°C till their further use for RNA extraction. Tips and eppendrfoff's tubes were treated with Di-ethyl pyro-carbonate (DEPC).

The RNA was extracted from the fragmented frozen *longissimus dorsi* muscle (120 mg) of adult animals of both the breeds. TRIzol™ (QIAZEN) reagent was used to isolate RNA from *longissimus dorsi* muscle. The tissue was homogenized by mixing with 2.0 ml of TRIzol and 400 µl of chloroform. Then, isopropanol (HIMEDIA) was subsequently used to precipitate the homogenized tissues. Later, 1 ml of 75% ethanol was used to wash the precipitated pellet. Immediately, followed by extraction, isolated samples of RNA were stored at -80°C. The genomic DNA impurities from RNA samples (25 µg) were eliminated with the RNase-free DNase set (QIAGEN, Hilden, Germany) and RNA was purified with the RNeasy mini kit according to the manufacturer's guidelines (QIAGEN, Hilden, Germany). The quantity of RNA was assessed by the thermo scientific Nanodrop one and quality was judged by automated capillary gel electrophoresis according to manufacturer's guidelines (Agilent Technologies Ireland, Dublin, Ireland). The ratios

of 28S/18S for the RNA samples were between 1.8 and 2.0 and the values for the integrity of RNA ranged from 8.0 to 10.0.

Quantitative Real Time PCR (qRT-PCR)

Primers for the qRT-PCR were constructed by the online Primer-3 software (Rozen and Skaletsky, 2000) and the information of the primers has been enlisted in Table 1. For the quantitative evaluation of mRNA transcript levels of *MyoD*, *Myf5*, *Myf6, Pax3* and *Pax7* genes; in indigenous and LWY, real-time qRT-PCR was performed using an (BIO RAD model CFX96^{TM} Optics Module real time PCR). To determine the quantity of transcripts of target genes EvaGreen (Biotium, USA) dye was used. Triplicate samples were used for the quantification by following the amplification conditions i.e. 95 °C for 10 min (initial denaturation), and then 39 cycles of 95 °C for 15 sec (denaturation) followed by 60 °C for 1 min (annealing and extending). The efficiency of real-time PCR was defined by the standard curve method. The amplified transcript levels of the target genes were compared with that of the *β-actin,* an endogenous control (Wang *et al*., 2003). mRNA transcript levels were quantified by the comparative C_T method. The results in terms of relative expressions have been expressed after normalising with the transcript levels of the endogenous reference (Erkens *et al*., 2006; Van Poucke *et al*., 2001).

Statistical analysis of the differential expression patterns of the MRFs

Statistical analysis of the differential expression patterns of the MRFs was done using ANOVA. The values have been expressed as mean ± SEM. $P<0.05$ has been set as statistical level of significance and subsequently Tukey's b-test was performed for analyzing the level of significance.

RESULTS AND DISCUSSION

Relative quantitative transcript levels of *MyoD, Myf5, Myf6, Pax7* and *Pax3* after their normalization with the transcript levels of the endogenous reference *β-actin*, have been investigated through Real Time qRT- PCR. In the present study, *MyoD* displayed significantly ($P<0.05$) higher quantitative transcript level in indigenous pigs than in LWY (Fig. 1, Table 2).

Both *Myf6* and *Myf5* did not indicate any statistically significant difference among themselves in the respective breeds during postnatal development (Fig. 1). Further, a statistically significant ($P<0.05$) higher expressions have been observed in the LWY as compared to indigenous pigs (Table 3 and Table 4). No statistically significant difference in the transcript levels of both *Myf6* and *Myf5* between the breeds during postnatal development has been supported with the findings in the knockout mouse with *MyoD* and *Myf6* or *Myf5* (Kassar-Duchossoy *et al*., 2004). Moreover, significantly similar transcript levels of

Table 1: Primer sequences of *MyoD, Myf5, Myf6, Pax3, Pax7, β- actin* for PCR

Primer	Primer Sequence	Product Size	Tm	Gene bank ID
MyoD	F 5'-TGCAAACGCAAGACCACTAA - 3'	127 bp	55	NM_001002824.1
	R 5'- GCTGATTCGGGTTGCTAGAC -3'			
Pax7	F 5' – GGCAGAGGATCTTGGAGACA – 3'	144 bp	55	AY653213.1
	R 5' – TGGGTGGGGTTTTCATCAAT – 3'			
Myf5	F 5' – CCGACACAGCTTGTGGAATA - 3'	128 bp	55	XM_001924362.2
	R 5' – GCCAATCAACTGATGGCTTT – 3'			
Myf6	F 5' – ATCTTGAGGGTGCGGATTTC – 3'	108 bp	62	XM_003481764
	R 5'- CAATGTTTGTCCCTCCTTCCT – 3'			
Pax3	F 5' – ATCGGCTAATCCTGACACATGC–3'	130 bp	54	AY579430.1
	R 5' – ACGGTGGGAAACTTTTGATG – 3'			
β- actin	F 5'- GACATCCGCAAGGACCTCTA - 3'	157 bp	60	XM_003124280
	R 5'- ACACGGAGTACTTGCGCTCT - 3'			

Table 2: Validation of expression of *MyoD* mRNA using real time PCR (SYBR green)

Group	*MyoD* (Average C_t)	β-actin (Average C_t)	Δ CT (C_t *MyoD* - C_t β-actin)	ΔΔCT ΔC_t of treated –ΔC_t of untreated)	Fold change
LWY	28.72±0.82	22.47±0.18	6.25±0.75	0±0.75	1
Indigenous	31.48±1.65	22.65±0.16	8.83±1.01	-0.64±1.01	1.56*

Values are Mean ± SE. Mean's bearing superscript *differ significantly at p <0.05.

Table 3: Validation of expression of *Myf5* mRNA using real time PCR (SYBR green)

Group	*Myf5* (Average C_t)	β-actin (Average C_t)	Δ CT (C_t *Myf5* - C_t β-actin)	ΔΔCT| ΔC_t of treated –ΔC_t of untreated)	Fold change
LWY	26.68±1.166	22.58±0.17	4.1±1.12	0±1.12	1
Indigenous	33.29±0.22	19.37±0.04	13.92±3.90	6.07±0.3.90	0.014*

Values are Mean ± SE. Mean's bearing superscript *differ significantly at p <0.05.

Table 4: Validation of expression of *Myf6* mRNA using real time PCR (SYBR green)

Group	*Myf6* (Average C_t)	β-actin (Average C_t)	Δ CT (C_t *Myf6* - C_t β- actin)	ΔΔCT ΔC_t of treated –ΔC_t of untreated)	Fold change
Indigenous	34.92±0.63	24.40±0.05	10.52±0.16	7.74±0.15	0.0046*

Values are Mean ± SE. Mean's bearing superscript *differ significantly at p <0.05.

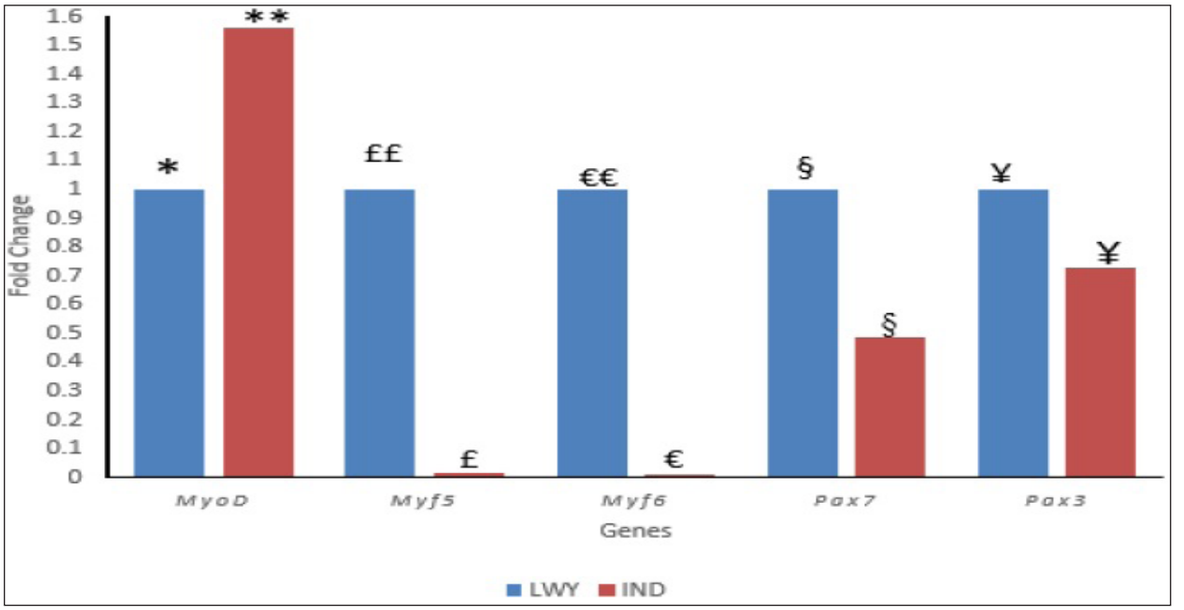

Fig. 1: Relative transcript level (RQ) of *MyoD*, *Myf5*, *Myf6*, *Pax7*, *and Pax3 in MRFs in* postnatal *longissimus dorsi* muscles of Large White Yorkshire and indigenous pigs. *RQ* has been normalized with the transcript levels of the endogenous references (β- actin) and keeping Large White Yorkshire as reference sample. Bars with different superscripts show significant differences in the transcript levels between the two breeds (p<0.05). Values are expressed as mean ± SEM

Myf5 and *Myf6* genes in both the breeds indicate towards their close vicinity on SSC5. It is a well-documented fact that postnatal muscle growth in pigs is a collective result of association of satellite stem cells and hypertrophic mechanisms (Ishido *et al*., 2008). In single, double and triple mutant mice an up- stream activity of *Myf6* with *MyoD* gene has been reported (Ropka-Molik *et al*., 2011). *Myf6* is a plentifully expressed myogenic factor in the postnatal muscles and it quantitatively dominates over the transcripts of *MyoD* family (White *et al.,* 2010). The raised *Myf6,* mRNA and protein levels are related with the bigger myofiber size (Yin *et al.,* 2013) and higher mean fiber area (Hespel *et al*., 2001). *Myf6* has dominant expression levels in postnatal mature fibers (Maak *et al*., 2006). Polymorphism in the promoter region and exon 1 of *Myf6* is reported to be significantly related with the weight of *longissimus dorsi* muscle and daily weight gain (Wyszynska-Koko and Kuryl, 2004). Significantly similar transcript levels of *Myf6* in both the breeds may help in explaining the presence of fast-growing fiber types in LWY and better meat quality related properties of indigeneous.

Non-significantly, lower expression of *Pax3* and *Pax7* have been recorded in indigenous pigs (Fig. 1 Table 5 & Table 6). These genes are reported to play critical role during fetal development and their proteins are essential for renewal and maintenance of stem cells. Moreover, it has been reported that levels of *Pax7* transcript depend on type of muscle and breed (Ropka-Molik *et al.,* 2011). Higher muscularity in Pietrain pigs is suggestive of higher expression of *MyoD* and *Pax7* (Ropka-Molik *et al*., 2011). Such findings are parallel to our results, since the animals of LWY breed showed higher transcript levels than indigenous pigs which may support higher skeletal muscle regeneration potential of LWY pigs. That's why these genes are targeted as candidate genes for meat production in pigs.

Myofibrils encompass actin and myosin as the main proteins in thin and thick filaments (Zhang, 2009). Major protein in the thick filaments which influences the type of muscle fiber is myosin. The type of the muscle fibers is reported to be responsible for the color, stability, and tenderness of meet.

CONCLUSION

Enhancements in body growth rate and quality of pork are amongst the top urgencies of breeding plans. Indigenous breeds have good meat quality, high tenderness and juiciness but lags in body growth rate as compare to western breeds. Constant fall in the quality and carcass characteristics have guided the breeders to improve such parameters. LWY is a rapidly-growing breed with large heavy muscular body and higher muscle fibers in the carcass with respect to indigenous breeds.

Table 5: Validation of expression of *Pax7* mRNA using real time PCR (SYBR green)

Group	*Pax7* (Average C_t)	β-actin (Average C_t)	Δ CT (C_t*Pax7*- C_t β-actin)	ΔΔCT ΔC_t of treated – ΔC_t of untreated)	Fold change
LWY	27.18±4.36	23.46±0.24	3.72±4.43	0±4.34	1
Indigenous	23.51±2.50	19.21±0.23	4.3±2.30	1.05±2.30	0.48

Values are Mean ± SE. Mean's bearing superscript *differ significantly at p <0.05.

Table 6: Validation of expression of *Pax3* mRNA using real time PCR (SYBR green)

Group	*Pax3* (Average C_t)	β-actin (Average C_t)	Δ CT (C_t*Pax3*- C_t β-actin)	ΔΔCT| ΔC_t of treated – ΔC_t of untreated)	Fold change
LWY	27.14±1.37	22.52±0.09	4.62±1.35	0±1.35	1
Indigenous	26.43±1.36	21.91±0.27	4.52±1.43	0.45±1.43	0.729

Values are Mean ± SE. Mean's bearing superscript *differ significantly at p <0.05.

Our study on the comparative quantitative transcript levels of MRFs in LWY and indigenous pigs introduced them as candidate genes which are associated with body growth and quality of pork. Till now very few studies covering transcript richness of the MRFs in indigenous pigs have been performed. Current experimental study is one of the innovator expression studies in indigenous pigs. Findings of current study can be useful in understanding the genetic basis for myogenesis in postnatal adult muscles of indigenous pigs and in the breeds with low meat fat ratio. These findings can be utilized as the basis to plan further researches to completely understand the detailed signaling pathways for the transcriptional activation of target genes.

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