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Studies on expression of different virulence genes of *Pasteurella multocida*

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ABSTRACT

Pasteurella multocida is the causative agent of a wide range of diseases in avian and mammalian hosts. Different adhesin and membrane proteins play role in the pathogenesis of the disease. In the present study, the relative expression of 5 different virulence genes (*plpE, ptfA, tbpA, hgbA* and *fhaB1)* from *Pasteurella multocida B:2* grown in iron rich and iron limiting media was measured using real time PCR employing SYBR green chemistry. The expression of *tbpA, hgbA, plpE* and *fhaB1* was found to be significantly upregulated by 4, 2.3, 1.3, 2.3 folds, respectively, under iron limiting conditions. In contrast, the expression of gene *ptfA* was significantly down regulated (0.4 fold) as compared to organism grown in normal medium.

Key words: Iron limitation, *Pasteurella multocida,* Real Time PCR, Virulence gene expression

Pasteurella multocida is a Gram negative bacterial pathogen known to affect a wide range of domestic as well as wild animals and avian species (Hunt *et al.* 2000) thereby leading to huge economic losses. The organism is divided into 5 capsular (A, B, D, E and F) and 16 somatic (1–16) types. The pathogenicity of bacteria is attributed to various virulence factors. The recognized virulence factors of *P. multocida* include diverse adhesins (e.g. filamentous haemagglutinin, type 4 fimbriae, and Flp pilin), toxins (dermonecrotic toxin), siderophores (e.g. iron acquisition proteins), sialidases and membrane proteins (e.g. OmpA, OmpH etc.) (Marandi and Mittal 1997, Adler *et al.* 1999, Fuller *et al.* 2000, Hunt *et al.* 2000, Ewers *et al.* 2006). *plpE* encodes the cross protective surface antigen PlpE (Steen *et al.* 2010), *tbpA* for transferrin binding protein (TbpA) and *hgbA* for haemoglobin binding protein (HgbA) which are mainly involved in iron acquisition. The detection of prevalence of different virulence genes among *Pasteurella* is critical to effective prevention of *P. multocida* infections. It will also be interesting to study how these virulence genes are regulated inside the body and what factors affect their expression.

Iron is an essential nutrient for most organisms due to its important role in metabolic electron transport chains but the acquisition of this element is particularly difficult for pathogens attempting to establish infections in mammalian hosts in which free iron is virtually non-existent (Payne *et al*. 1978). The present study was aimed to study the effect of iron limitation on the level of expression on 5 important genes viz. *plpE, ptfA, tbpA, hgbA* and *fhaB1*from *P. multocida* serotype B:2.

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MATERIALS AND METHODS

Bacterial strains and DNA preparation: Pasteurella multocida (strain P52) was grown to log phase in brain-heart infusion (BHI) broth at 37°C. The culture was split into 2 parts and centrifuged at 6,000 g for 5 min at 4°C, washed with phosphate-buffered saline (pH 7.0) and centrifuged again. One pellet was resuspended in BHI broth and the other was resuspended in BHI broth containing 200 µM of iron chelator (2, 2'Bipyridyl). The resuspended cultures were incubated at 37°C in an orbital shaker incubator and fractions were removed at 10, 20, 30, 60, and 120 min intervals. These fractions were briefly centrifuged at 4°C and the pellets were immediately processed for extraction of RNA.

Relative gene expression of target genes using real-time PCR: The relative expression of different genes was studied by quantitative PCR using SYBR green chemistry. Total RNA from culture of organism collected at different time intervals was extracted using RNeasy Protect bacteria kit as per the manufacturer's protocol. One microgram of total RNA was used for cDNA synthesis using QuantiTect reverse Transcription Kit as per manufacturer's protocol.

The primers against the genes coding for Type 4 fimbrial gene (*ptfA)*, filamentous haemagglutinin $fhaB_1/B_2$, transferrin-binding protein (*tbpA),* haemoglobin-binding protein *(hgbA)* and *Pasteurella* lipoprotein E *(plpE)* were designed from the published gene references and got synthesized commercially (Table 1).

The optimal concentration of the primers to be used for

Table 1. Primers used for study of expression of virulence genes in *Pasteurella multocida*

Gene	Accession number	Primer Sequence (5'-3')	Amplicon size	Reference
plpE	EF219456.1	AGCCTTCTTCCGCTCCAATC ACTCGCATTTCCCACTTCTGA	209	This study
hgbA	AF331501.1	CATGGGAATCTGCTCGTTCG ATCCACCGCACTTTCCCTAA	180	This study
fhaB1/2	AF237928.1	GGCACTTACATCTTGCGGTA TTGTCGGAACACCAACAGAG	246	This study
ptfA	AY644678.1	TATCGTGCCGAGGTAGAACT AAACACCTTGAGCAGCGTTA	214	This study
tbpA	AJ558182.1	TTCGGACTTTCCGACGGTTT ATAACGCCCGCTCAAACTCA	159	This study

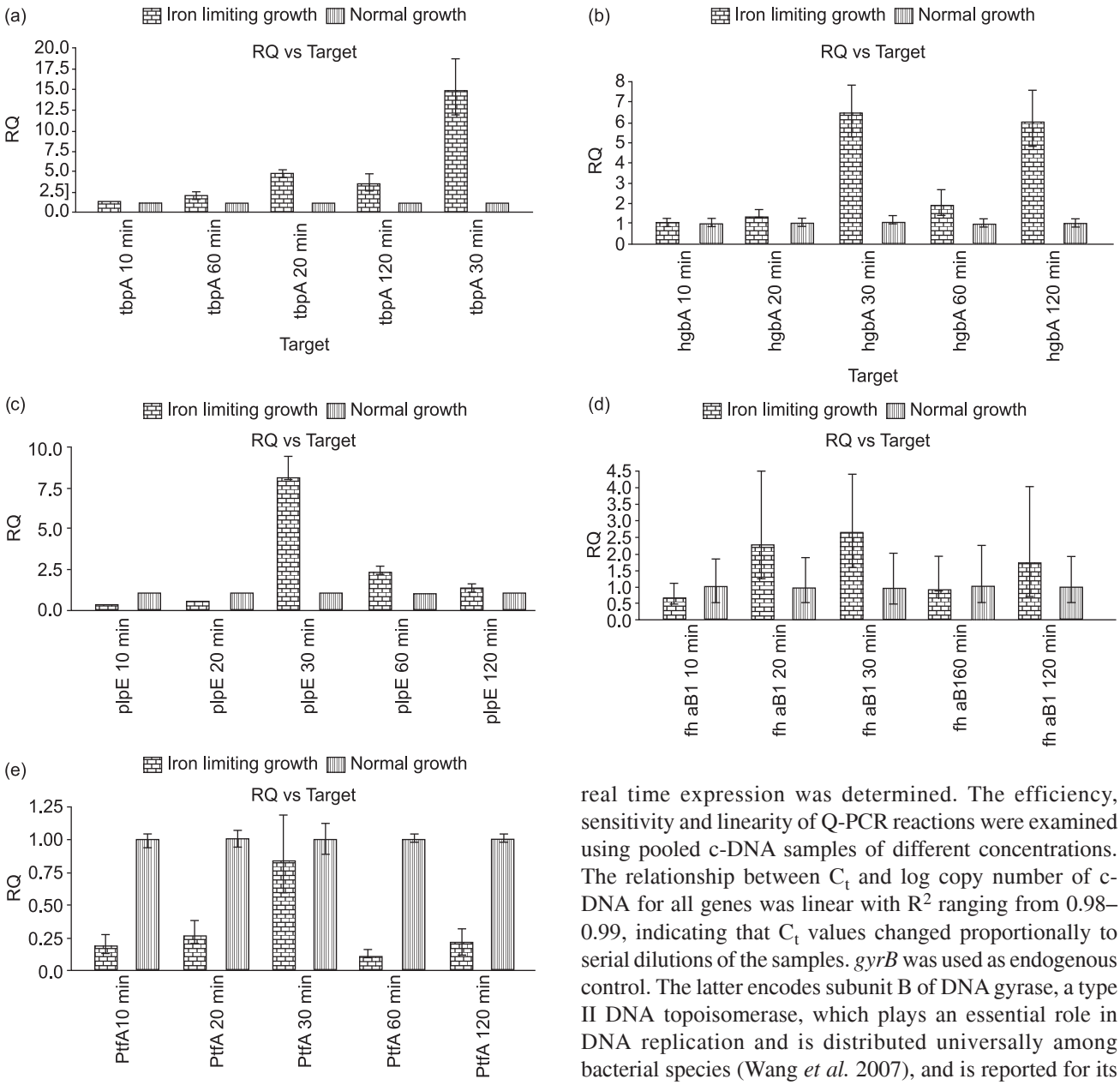

Fig. 1(a-e). Relative expression profiles of virulence genes of *P. multocida* at 10, 20, 30, 60 and 120 min after addition of the iron chelator. (**a**) *tbpA,* (**b**) *hgbA,* (**c**) *plpE,* (**d**) *fhaB1,* and (**e**) *ptfA*.

real time expression was determined. The efficiency, sensitivity and linearity of Q-PCR reactions were examined using pooled c-DNA samples of different concentrations. The relationship between C_t and log copy number of c-DNA for all genes was linear with R^2 ranging from 0.98–0.99, indicating that C_t values changed proportionally to serial dilutions of the samples. *gyrB* was used as endogenous control. The latter encodes subunit B of DNA gyrase, a type II DNA topoisomerase, which plays an essential role in DNA replication and is distributed universally among bacterial species (Wang *et al.* 2007), and is reported for its stable expression (Zhao *et al.* 2011).

The reaction was carried out in 20 µl final volume containing (2×) SYBR green master mix, 4 pmol of each primer and 5 µl of template cDNA from each aliquot and

nuclease free water to make volume 20 µl. The cycling conditions for the amplification were initial denaturation at 94°C for 10 min followed by 40 cycles each of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. The specificity of amplicons was determined by melt curve analysis (65°C–95°C at 0.1°/C).

Statistical analysis: The total expression ratio of the 5 genes of interest over a period of 2 h was tested for significance by independent t-test using SPSS software (version 20.0) for group wise comparison.

RESULTS AND DISCUSSION

Kinetics of virulence genes expression under iron limiting conditions: Relative expression study using Real time PCR by comparative C_T ($2^{-\Delta\Delta C}T$) method indicated that the expression levels of genes were altered with time and *tbpA, hgbA, plpE, fhaB1/2* genes were significantly up-regulated (Fig. 1a, 1b, 1c, 1d) and *PtfA* was down-regulated in response to diminished levels of iron (Fig.1e). The expression of the *tbpA* increased exponentially during 2 h under iron limiting conditions. The expression during first 10 min was similar to expression in normal growth condition. Thereafter the expression increased exponentially during the succeeding time intervals and peaked at 120 min. Upregulation of *tbpA* under iron limiting condition was also reported in other bacterial species (Nielsen *et al.* 2005). The increased expression of *tbpA* under iron limiting condition supports its role in iron acquisition of *Pasteurella multocida* from host. The initial expression of *plpE* after 10 min under iron limiting condition was lower as compared to normal growth condition but started increasing slowly after 20 min and peaked after 30 min. Besides, a drop in expression after 60 min, the level of expression started increasing slowly after 120 min. The level of expression of *hgbA* and *fhaB1* followed the similar pattern. The expression of both the genes started increasing after 10 min and peaked after 30 min. The expression decreased after 60 min but to a level higher than normal growth condition and the expression started increasing after 120 min. Up-regulation of *hgbA* under iron limiting condition during 2 h time period in the present study was also reported earlier (Bosch *et al.* 2002). There is scant literature as far as the expression of *fhaB1* gene in *Pasteurella multocida* is concerned, however, an increase in expression of *fhaB* in *Bordetella sp.* during growth in iron limiting conditions was also reported (Brickman *et al.* 2011). The upregulation of *fhaB1* gene under iron limiting conditions indicated the possible increased colonization and bacterial dispersion in host under natural state of iron starvation stress *in vivo*. Though expression of *ptfA* initiated after 10 min in response to iron limiting condition, attained peak level after 30 min but then slowly started to level off. At all the time periods, its expression was lower than that in the organisms grown under normal growth condition, thus indicating its down regulation. Previous studies on the gene expression of *P. multocida* also reported the transcriptional activation of several outer membrane proteins under nutrient limiting conditions (Paustian *et al.* 2002). But our observations are in contrast to those of Paustian *et al.* (2002), who reported upregulation of type IV fimbrial subunit gene.

Iron limiting condition has a significant effect on expression of virulence genes in *Pasteurella multocida*. All the genes under study (*plpE, hgbA, fhaB1 and tbpA*) were upregulated except *ptfA*. One notable feature of the data was an overall lack of dramatic changes in gene expression. None of the genes varied more than 6 fold between the experimental and control populations. Several factors may have contributed to this. The organisms were initially grown to log phase in BHI medium; therefore, the cells may have had a reserve of iron available for utilization immediately following re-suspension in minimal medium. It is also likely that the bacteria were growing at a generally reduced rate because of the nutrient limitations imposed by the minimal medium. The experimental design used in these experiments provides us with a useful system for studying the initial events of infection *in vitro* and provides strong evidence for the existence of specialized responses to defined sources of iron in *P. multocida*.

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