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Molecular typing of haemorrhagic septicaemia-associated *Pasteurella multocida* isolates from Pakistan and Thailand using multilocus sequence typing and pulsed-field gel electrophoresis

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Abstract

A comparative genetic study of 23 field isolates and vaccine strains of *Pasteurella multocida* associated with haemorrhagic septicaemia cases from Pakistan and Thailand was done using pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The MLST sequence type (ST) for all 20 of the 23 isolates tested was 122. The PFGE results showed one band difference between the Pakistani and the Thai isolates. Sequence type 122 is the dominant associated profile with haemorrhagic septicaemia (HS) cases in South Asia. The study supports the concept of using PFGE for short-term epidemiology and MLST for long-term epidemiology.

Introduction

Pasteurella multocida is a Gram-negative nonmotile and nonspore-forming rod or coccobacillus (Rimler and Rhoades, 1989). It is serologically classified into five Carter capsular groups, namely A, B, D, E and F (Carter, 1963; Rimler and Rhoades, 1987) and sixteen Heddleston somatic types (Heddleston et al., 1972). Different serotypes of *P. multocida* are associated with a variety of diseases in animals, such as fowl cholera (mainly serotypes A:1, A:3 and A:4) and atrophic rhinitis in pigs (toxigenic strains of serogroup D) (De Alwis, 1999, pp. 11–27).

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease in cattle and buffaloes associated with strains of serotypes B:2 (Asian serotype) and E:2 (African serotype) of the bacterium *P. multocida* (De Alwis, 1999, pp. 11–27), however HS-associated B:2 serotypes may occur in Africa, and E:2 serotypes may be found in Asia (Dziva et al., 2008).

Asia and Africa are currently the global geographic regions in which HS occurs with the highest prevalence and has the greatest economic importance (Benkirane and De Alwis, 2002). This is attributed to pronounced changes in weather between seasons, including the monsoon, debility caused by seasonal scarcity of fodder and pressure of work (draught animals) (Benkirane and De Alwis, 2002).

Haemorrhagic septicaemia has been reported to be the most important bacterial disease of cattle and buffaloes in Pakistan, and is considered a disease of great economic importance (Benkirane and De Alwis, 2002). In 1978, Pakistan reported that 34.4% of all deaths in buffaloes and cattle were due to HS and the estimated annual economic losses were 1.89 billion rupees (Benkirane and De Alwis,

2002). In Thailand, HS ranks highly on the list of economically significant diseases of cattle and buffaloes (Patten et al., 1993).

Molecular typing of different bacterial isolates is an essential molecular epidemiological tool. Different DNA typing methods such as restriction endonuclease analysis (REA), ribotyping, PCR methods, such as Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, Repetitive Extragenic Palindromic (REP) PCR, Random Amplification of Polymorphic DNA (RAPD) PCR and pulsed-field gel electrophoresis (PFGE) have been used for genotyping of *P. multocida* (Blackall and Miflin, 2000). Multi locus enzyme electrophoresis (MLEE) (Blackall et al., 1998) and multi locus sequence typing (MLST) (Subaaharan et al., 2010) have also been used for molecular epidemiology. MLST is a portable and reproducible sequence based approach to molecular genotyping which facilitates the comparison of results from different laboratories (Maiden et al., 1998). PFGE has shown a great discriminatory power to investigate individual outbreaks in one geographic area so it is considered a gold standard for short term epidemiology (Maiden et al., 1998). On the other hand, MLST has been claimed to be the gold standard for studying long term (global) epidemiology (Maiden et al., 1998).

A MLST scheme has been developed for *P. multocida* called the Rural Industries Research and Development Corporation (RIRDC) *P. multocida* MLST scheme (Subaaharan et al., 2010). This database was developed initially for avian isolates and all the primer information, PCR conditions, allele sequences, sequence types and isolate information are available at the RIRDC *P. multocida* MLST website (http://pubmlst.org/pmultocida_rirdc/) sited at the department of Zoology, University of Oxford (Jolley et al., 2004). Presently, this database contains DNA sequences of *P. multocida* isolates from many hosts and from different pasteurelloses. Another scheme exists, called the *P. multocida* multi-host MLST scheme but it is not accepting submissions nowadays. (http://pubmlst.org/pmultocida_multihost/).

In this study we investigated the relatedness of HS-associated isolates of *P. multocida* within and between two HS-endemic countries in South Asia, namely Thailand and Pakistan. We also compared the discriminatory power of MLST and PFGE for the purpose of detecting genetic differences between these various isolates.

Materials and methods

A total of 20 field isolates and 3 vaccine strains previously identified as *P. multocida* were used in this study ($n = 23$). Twelve Pakistani field isolates and the Peshawar vaccine strain were collected from the National Veterinary Laboratory (NVL), Islamabad. The Lahore vaccine strain (B:2 serotype) was collected from the Veterinary Research Institute in Lahore. An additional 9 isolates (8 field strains and 1 vaccine strain) were received from the Department of Livestock Development (DLD), Ministry of Agriculture and Cooperatives, Thailand (Table 1).

Biochemical characterisation

Isolates 1–20 were characterised biochemically using the Microbact Gnb 24E tray (Oxoid, UK, Cat# MB1131A), according to the manufacturer's instructions. Briefly, each isolate was grown on brain heart infusion (BHI) agar (Merck Chemicals) for 24 h at 37 °C and then 2–3 pure colonies were inoculated in 5 mL sterile saline (0.85%) containing 5 drops of sterile foetal bovine serum. Motility was checked for all isolates by stab inoculation into a Tryptone Soya slant (semisolid medium) (Oxoid, UK, Cat# CM0131). The results were interpreted after incubation for 48 h at 37 °C.

Each isolate was also tested for oxidase production using oxidase test strips (Oxoid, UK, Cat#MB0266), and catalase production using hydrogen peroxide.

An octal number was assigned to each isolate and then interpreted using the Microbact computer aided identification package (MB1244A). The results of oxidase test, motility and nitrate reduction were added to this octal number to produce a nine digit code which was entered into the software program.

DNA extraction

Genomic DNA of *P. multocida* was extracted using the Purelink genomic DNA Kit (Invitrogen Cat# K182001). The protocol for Gram negative bacteria as described in the kit was followed for extractions.

Molecular identification of the bacterial isolates

The two primers KMT1T7 and KMT1SP6 (Table 2) (Townsend et al., 1998), were used to confirm all isolates as *P. multocida*, by amplification of a 460 bp amplicon.

The primer pair, KTT72 and KTSP61 (Table 2) amplifies a 620 bp fragment in all isolates of *P. multocida* serogroup B, serotypes 2 or 5 (Townsend et al., 1998).

A multiplex PCR combining *P. multocida*-specific and HS-associated serotypes B:2 or B:5-specific reactions was used. The total reaction volume was 20 µL containing 10 µL of 2 × Master Mix (Roche cat# 11636103001) and 1.25 µL of each primer (10 µmol/L) for the serogroup B HS-specific test and 0.75 µL of each primer (10 µmol/L) of the *P. multocida* specific test. Two µL of DNA template and 4 µL of deionized water were added to each reaction. Cycling parameters were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. Ten µL of the reaction volume was electrophoresed through a 2% DNA grade agarose gel and was visualised by UV transillumination.

Multiplex PCR assay for capsular typing of *P. multocida*

The capsular typing primers (Table 2) were used to investigate the capsular type of all field isolates used in this study. Reagent concentrations and cycling parameters were as per Townsend et al., 2001. Again, 10 µL of the reaction volume was electrophoresed through a 2% DNA grade agarose gel and visualised by UV transillumination.

Multi locus sequence typing (MLST)

The seven genes used in the *P. multocida* MLST scheme are adenylate kinase (*adk*), esterase (*est*), mannose-6-phosphate isomerase (*pmi*), glucose-6-phosphate dehydrogenase (*zwf*), malate dehydrogenase (*mdh*), glutamate dehydrogenase (*gdh*) and phosphor glucose isomerase (*pgi*) (Subaaharan et al., 2010) (Table 2). The MLST scheme was performed on 20 isolates according to the protocol of Subaaharan et al. (2010).

All purified products were sequenced at the DNA Core Facility at the Centre for Applied Molecular Biology (CAMB), Ministry of Science and Technology, Lahore, Pakistan. For each amplified fragment, 2 sequencing reactions were performed separately, one with the forward and one with the reverse primer. The raw sequence chromatograms were checked and edited by Sequence Scanner v1.0 software. The forward and reverse complimented sequences were aligned together in sequence alignment software (Bioedit version 7.0.9.0) (Hall, 1999).

For each locus of each isolate, the sequence was checked in the Blast tool of the pubmlst.org database to determine the identity of the allele. A seven-digit allelic profile was assigned for each isolate. The sequence type (ST) was then identified according to the seven digit allelic profile.

Pulsed field gel electrophoresis (PFGE)

P. multocida isolates were cultivated in brain heart infusion broth. The bacterial suspension in Tris–EDTA buffer supplemented with 1 mg/mL of lysozyme, adjusted to 4 U (McFarland value) was mixed with an equal volume of 2% low melting point agarose gel (Bio-Rad, USA). This was incubated at 37 °C for 4 h in a buffer containing 6 mmol/L Tris HCl, 100 mmol/L EDTA, 1 mol/L NaCl, 0.5% (w/v) Brij58, 0.2% sodium deoxycholate and 0.5% lauroyl sarcosine supplemented with 1 mg/mL of lysozyme, then incubated at 55 °C overnight in a solution containing 0.25 mol/L EDTA, 20 mmol/L NaCl, 1% lauroyl sarcosine and 1 mg/mL of proteinase K, after which it was washed 3 times in Tris–EDTA buffer at 4 °C for 1 h.

Total bacterial DNA was digested with 30 U ApaI (New England BioLabs, USA), following the digestion conditions indicated by the producer. The DNA fragments which resulted were separated by a CHEF – DR III (Bio Rad, USA) system in 1% agarose (Bio Rad, USA). The electrophoresis conditions were as follows: Tris–borate-EDTA 0.5 × buffer, at 14 °C, 6 V/cm, at an angle of 120° and a linear ramp. For the separation of the macro-restriction ApaI fragments, the migration program lasted 22 h, with a pulse spacing of 1–25 s.

Results

Biochemical identification

All 20 checked isolates were non-motile, produced oxidase and catalase, produced indole and reduced nitrate (NO_3^-). They were β-galactosidase, lysine decarboxylase, hydrogen sulphide, Voges-Proskauer, citrate, TDA (tryptophan deaminase), l-arginine, gelatin, malonate and urease negative. They fermented glucose, mannitol, sorbitol and sucrose; however, they did not ferment inositol, rhamnose, lactose, arabinose, adonitol, raffinose or salicin, nor did they ferment xylose, with the single exception of the Thailand D isolate.

Only five isolates (Karachi 2, Peshawar vaccine strain, Bhakkar, Attock and Islamabad 1) were ornithine decarboxylase negative.

The Microbact™ Computer Aided Identification Package identified these 20 isolates as *P. multocida* with ⩾99.98% certainty.

Multiplex PCR

All 23 isolates produced 2 bands of 460 bp and 620 bp, which is consistent with HS-associated *P. multocida* strains. In the capsular typing multiplex PCR, all isolates produced one band at 760 bp which represents capsular type B.

MLST amplified products

All the amplicons were sequenced and the results were the same for each gene in all 20 isolates tested. The sequences of the genes *adk, est, mdh, gdh, pgi, pmi* and *zwf* corresponded to the RIRDC MLST database allele identifiers 23, 37, 4, 2, 17, 21 and 17, respectively. This allelic profile corresponds with sequence type 122. These isolates' results have been submitted to the *P. multocida* RIRDC MLST database and have been accepted and given the isolate identification numbers 539–558 (Table 1).

Pulsed field gel electrophoresis (PFGE)

Fig. 1 shows the PFGE results for 14 isolates from Thailand and Pakistan. This figure shows 3 Thai isolates which were typed only by PFGE namely, Thailand G and H and Thai HS vaccine strain in

lanes 7, 8 and 9, respectively. There is one band difference between the Pakistani isolates and the Thai isolates.

Discussion

All isolates had virtually identical biochemical properties, which confirmed their identity as *P. multocida*. However, a simple aetiological diagnosis based on phenotypic characteristics alone lacks the ability to detect more subtle differences between isolates. Tests with the ability to differentiate between isolates based on their genotype can be used to achieve greater discriminatory power. The ability to discern subtle genetic differences between isolates of *P. multocida* can help to investigate many questions regarding the diversity of isolates collected during disease outbreaks and this will be particularly relevant in studies on the short term epidemiology of HS in particular HS-endemic localities. Furthermore, genetic characterisation enables the ready comparison of isolates recovered from different HS outbreaks in different geographic areas and over time, thus providing useful data for long term epidemiology studies.

Knowledge of the genetic diversity of HS-associated *P. multocida* isolates in particular localities enables the rational selection of isolates for vaccine production and distribution. Monitoring the evolving genetic and clinical virulence characteristics of HS-associated *P. multocida* isolates over time may enable the identification of genes responsible for virulence, using the power of comparative genomics. Such information would help with the development of more effective vaccines.

MLST investigates the sequence of just seven housekeeping genes, where mutations are known to accumulate very slowly (Maiden et al., 1998). In comparison, PFGE can also investigate uncharacterized regions of the entire genome that may be highly variable, and therefore may have a stronger discriminating power compared to MLST for microvariation between isolates circulating in the same geographic area (Maiden et al., 1998). This had been reported before for other

microorganisms such as *Pseudomonas aeruginosa*, *Salmonella enterica* serovar

Typhimurium, *Staphylococcus aureus* and *Escherichia coli* (Noller et al., 2003; Fakhr et al., 2005; Giske et al., 2006; Hallin et al., 2007; Johnson et al., 2007). On the other hand, other studies showed that MLST had a greater discriminatory power than PFGE for *Vibrio cholera* and *E. coli* (Kotetishvili et al., 2003; Nemoy et al., 2005).

The existence of the RIRDC *P. multocida* MLST database enables the ready sharing of isolate details and sequencing results, and this provides scope to examine genetic and epidemiological patterns in HS across the entire region of South Asia.

There were 565 different isolates on the RIRDC *P. multocida* MLST database as of 30 November 2012. These isolates represent 276 different profiles, from 41 hosts, across 33 countries and associated with 27 diseases. Although initially this database targeted avian isolates, now ruminant-derived isolates make up a high proportion of database entries with cattle, buffaloes and other bovines representing 198/565 (35%) isolates. Sequence type 122 was recorded in 55/565 (9.7%) cases, of which 46 (8.1%) were recovered from ruminants diagnosed with HS. For the remaining 9 cases in which ST 122 was identified, the disease was not assigned. Only three isolates obtained from HS-affected ruminants were not ST 122: with one instance each of ST 8 (Indonesia), ST 51 (Country unassigned) and ST 129 (Sri Lanka). It is clear from these statistics that more isolates from HS cases need to be obtained and genotyped according to the MLST scheme, to provide a more accurate representation of the real situation in the field.

In our study, 20 isolates from Pakistan and Thailand were all ST 122, according to the MLST method, whereas using the PFGE method, a single band difference was demonstrable between the isolates obtained from Pakistan and those obtained from Thailand. Isolates from Pakistan which had been collected from different geographical areas, climate zones and from outbreaks in different years, were indistinguishable by MLST or PFGE. Likewise, all Thai isolates in this study were indistinguishable from one another by MLST or PFGE. This supports the idea that one locally sourced vaccine strain may be sufficient to meet the needs of each country. However, a method with greater discriminatory power is needed to prove the diversity or unity of these various isolates.

The MLST results for 20 isolates were also consistent with the reported sequence type of HS-associated isolates from other countries in the region, namely India, Myanmar, East Timor and Indonesia. Since ST 122 appears to be the predominant HS-associated strain of *P. multocida* in Thailand, India, Indonesia, East Timor, Myanmar and Pakistan, there is potential for region-wide strategies to be adopted aimed at regional control of HS.

PFGE showed that Pakistani isolates differed slightly from the Thai isolates: they lacked one band compared with the Thai isolates. The Thai vaccine strain had the same profile as the Thai field isolates, and the Pakistani vaccine strains had the same profile as the Pakistani field isolates. These results lend support to the policy of using locally sourced HS-associated *P. multocida* isolates to manufacture vaccines in Pakistan and Thailand.

Conclusions

The difference between the PFGE profile of the Thai isolates and the Pakistani isolates should be further studied to determine how these genetic variations could affect the efficacy of using one HS vaccine for the whole region. It is also clear that both MLST and PFGE have pros and cons, and in reality, a technique that can combine the advantages of both of them and decrease the disadvantages is needed. Fortunately, modern molecular methods, such as next generation sequencing (NGS) now exist which can potentially meet this need. NGS can provide the precision, reproducibility and portability of DNA sequencing with the whole-genome evaluation characteristics of PFGE and therefore may eventually replace them both.

Conflict of interest

Acknowledgments

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Fig. 1. PFGE results for fourteen isolates. Lane M: Lambda PFG marker. Lane 1: Thailand A. Lane 2: Thailand B. Lane 3: Thailand C. Lane 4: Thailand D Lane 5: Thailand E Lane 6: Thailand F. Lane 7: Thailand G. Lane 8: Thailand H. Lane 9: Thai HS vaccine strain. Lane 10: Attock. Lane 11: Karachi 3. Lane 12: Peshawar. Lane 13: Peshawar vaccine. Lane 14: Lahore vaccine.
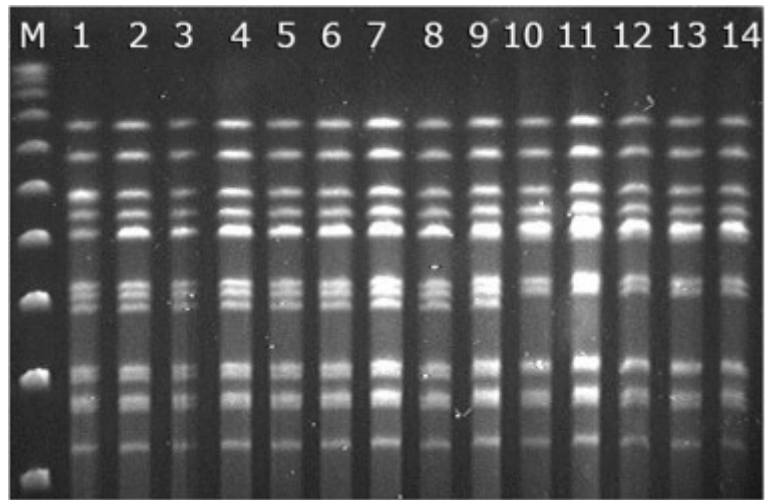
Table 1. Isolates used in the study.

Isolate number	Isolate ID	District	Province	Country	Location coordinates	Species	Year	RIRDC MLST accession number	PFGE results (Fig. 1)
1	Sahiwal	Sahiwal	Punjab	Pakistan	30.66°N, 73.11°E	Buffalo	2008	545	ND
2	Bhakkar	Bhakkar	Punjab	Pakistan	31.63°N, 71.07°E	Cattle	2008	548	ND
3	Attock	Attock	Punjab	Pakistan	33.91°N, 72.31°E	Cattle	2010	546	Lane 10
4	Karachi 1	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Cattle	2007	551	ND
5	Karachi 2	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Buffalo	2011	552	ND
6	Karachi 3	Karachi	Sindh	Pakistan	24.86°N, 67.01°E	Buffalo	2011	553	Lane 11
7	Peshawar	Peshawar	Khyber Pakhtunkhwa	Pakistan	34.02°N, 71.58°E	Buffalo	2011	556	Lane 12
8	Islamabad 1	Islamabad	Islamabad capital territory	Pakisatn	33.72°N, 73.07°E	Wild buffalo	2011	554	ND
9	Islamabad 2	Islamabad	Islamabad capital territory	Pakistan	33.72°N, 73.07°E	Buffalo	2012	555	ND
10	Jhelum	Jhelum	Punjab	Pakistan	32.93°N, 73.73°E	Buffalo	2009	547	ND
11	Taxila 1	Rawalpindi	Punjab	Pakistan	33.75°N, 72.79°E	Buffalo	2012	549	ND
12	Taxila 2	Rawalpindi	Punjab	Pakistan	33.75°N, 72.79°E	Buffalo	2012	550	ND
13	Lahore vaccine strain	Lahore	Punjab	Pakistan	31.55°N, 74.34°E	Buffalo	2011	558	Lane 14
14	Peshawar vaccine strain	Peshawar	Khyber Pakhtunkhwa	Pakistan	34.02°N, 71.58°E		2011	557	Lane 13
15	Thailand A	Thung song	Nakhon Si Thammarat	Thailand	8.16°N, 99.68°E	Buffalo	2006	539	Lane 1
16	Thailand B	Mueang Phitsanulok	Phitsanulok	Thailand	16.82°N, 100.26°E	Buffalo	2011	540	Lane 2
17	Thailand C	Rong Kham	Kalasin	Thailand	16.27°N, 103.74°E	Buffalo	2006	541	Lane 3
18	Thailand D	Phanat Nikhom	Chonburi	Thailand	13.45°N, 101.18°E	Buffalo	2009	542	Lane 4
19	Thailand E	Chiang Dao	Chiang Mai	Thailand	19.37°N, 98.96°E	Buffalo	2009	543	Lane 5
20	Thailand F	Mueang Lamphun	Lamphun	Thailand	18.58°N, 99.02°E	Buffalo	2011	544	Lane 6
21	Thailand G	Warin Chamrap	Ubon Ratchathani	Thailand	15.20°N, 104.87°E	Buffalo	2008	ND	Lane 7
22	Thailand H	Mueang Khon Kaen	Khon Kaen	Thailand	16.44°N, 102.84°E	Buffalo	2010	ND	Lane 8
23	Thai HS vaccine strain	NA	NA	Thailand	NA	Buffalo	NA	ND	Lane 9

ND = not done.

NA = not applicable.

Table 2. Primer sequences for molecular identification of the bacterial isolates (multiplex

PCR), *Pasteurella multocida* capsular typing PCR reactions and MLST scheme.

Molecular identification of the bacterial isolates (multiplex PCR)

Name	Primer sequence	Primer sequence	Amplicon size (bp)	Ref.
Pasteurella multocida specific PCR	KMT1T7	KMT1SP6	460	(Townsend et al., 1998)
	ATCCGCTATTTACCCAGTGG	GCTGTAAACGAACTCGCCAC		
HS-associated serotypes B:2 or B:5 specific PCR	KTT72	KTSP61	620	(Townsend et al., 1998)
	AGGCTCGTTTGGATTATGAAG	ATCCGCTAACACACTCTC		

Pasteurella multocida capsular typing

Capsular type	Forward primer	Reverse primer	Amplicon size (bp)	Ref.
A	TGCCAAAATCGCAGTGAG	TTGCCATCATTGTCAGTG	1044	(Townsend et al., 2001)
B	CATTTATCCAAGCTCCACC	GCCCGAGAGTTTCAATCC	760	(Townsend et al., 2001)
D	TTACAAAAGAAAGACTAGGAGCCC	CATCTACCCACTCAACCATATCAG	657	(Townsend et al., 2001)
E	TCCGCAGAAAATTATTGACTC	GCTTGCTGCTTGATTTTGTC	511	(Townsend et al., 2001)
F	AATCGGAGAACGCAGAAATCAG	TTCCGCCGTCAATTACTCTG	851	tim

MLST scheme

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Ref.
adk	GGGGAAAGGGACACAAGC	TTTTTCGTCCCGTCTAAGC	570	(Subaaharan et al., 2010)
est	TCTGGCAAAAGATGTTGTCG	CCAAATTCTTGGTTGGTTGG	641	(Subaaharan et al., 2010)

pmi	GCCTTAACAAGTCCCATT CG	TGCCTTGAGACAGGGTA AGC	739	(Subaaharan et al., 2010)
zwf-1	AATCGGTCGTTTGACTGA GC	TGCTTCACCTTCAACTGT GC	808	(Subaaharan et al., 2010)
mdh	GGAAAACCGGTAATGGAA GG	ATTTCGGGATCAGGGTTA GC	620	(Subaaharan et al., 2010)
gdh	GCGGGTGATATTGGTGTA GG	ATCGACTTCTTCCGCAGA CC	702	(Subaaharan et al., 2010)
pgi	ACCACGCTATTTTTGGTTG C	ATGGCACAACCTCTTTCA CC	784	(Subaaharan et al., 2010)
zwf-2	TGTTAGGTGTGGCAAGAA CG	TTGCAACAAATGGTTTTG GA	614	(Subaaharan et al., 2010)