“Pitfalls” of bacterial identification methods

Sunita D Singh and Parvathi.J.R

***Abstract*—** The conventional approaches in bacterial identification involve phenotypic methods fully based on colony morphology and biochemical features. The increasing demand for rapid assays has been decisive in making molecular assay, the prime choice for detection and diagnosis. Both these practices have their share of advantages and disadvantages leading to a scenario wherein authentication is probable with stringent work culture and operation procedures. A multi-disciplinary approach from microbiology, bioinformatics and molecular biology to “mark” a candidate microbe has become a necessity of the hour.

***Keywords*—**Biochemical tests, Phase variation, PCR-based techniques, Molecular assays

I. Introduction

A looming global concern that causes 1.5 to 3 million deaths per year in health care are Nosocomial or Hospital acquired infections (HAI) [1]. According to the Nosocomial Infection Control Consortium (NICC) surveillance studies, *Escherichia coli* are among the most isolated organisms [2], [3] irrespective of their locations (ICU or non-ICU) within the hospital premises. Among the various HAIs, urinary tract infections (UTIs) are the most frequent [4]. Root causes of UTI are attributed to members of γ-*enterobacteriacea* [5] family with *E.coli* as the major player followed by *Citrobacter* and *Klebsiella* spp.

During such scenario, the identification of the causative pathogen becomes prerequisite for establishing any immediate control measures. *E.coli* is preliminary screened either on MacConkey agar (MAC) or Eosin Methylene Blue agar (EMB) and are “cited out” specifically on the basis of colony morphology and pigmentation [6]. Congruous colony morphovars by other bacterial strains in the same can hamper chances of confirmation of its identity. Further analysis involves two approaches, with one being entirely based on biochemical characterization and the other footed on molecular profiling. Conventional method or phenotypic assays involves battery of biochemical tests which confer a signature profile of the candidate microbe [7], [8]. Molecular methods target conserved regions within the core-genome of the bacterium for nucleic acid based amplification tests (NAATS) [9], [10]. A number of factors ranging from time duration to false negatives in the former and non-specific and ambiguous amplication in latter prove detrimental, when authentication of microbe is a necessity. Four causative agents of UTI under γ–*enterobacteriacea* family having colony resemblance in selective medium were targeted for this study. An investigation into the pitfalls of molecular and phenotypic methods can be analysed by checking the specificity of these approaches in differentiating among candidate species.

[1]Dr. Singh Sunita currently working as Associate Professor, Department of Biotechnology and Bioinformatics,Padmashree Dr. D. Y. Patil University. Plot No-50, Sector-15, C B D Belapur, Navi Mumbai-400614. Ph: 08879151573.Fax:91-22-39286176; e.mail: dr.sunitads@gmail.com.

[2]Parvathi J. R is Assistant Professor at Department of Biotechnology and Bioinformatics, Padmashree Dr. D. Y. Patil University. Plot No-50, Sector-15, C B D Belapur, Navi Mumbai-400614. Ph: 09833956533.Fax:91-22-39286176; e.mail: parvathijr@gmail.com

II. Methodology

A. Microbial Strains and Phenotypic tests

Lyophilized strains of *Citrobacter koseri* (MTCC 1658 /ATCC 8090), *Enterobacter aerogenes* (MTCC 111 /ATCC 13048), *Escherichia coli* (MTCC 433 /ATCC 15223) and *Klebsiella pneumoniae* (MTCC 432 /ATCC 33495) obtained from Microbial type culture collection (MTCC), Institute of Microbial technology (IMTECH, Chandigarh, India) were revived in nutrient broth. The cultures were streaked on MAC and EMB respectively; colony morphology and pigmentation were noted after overnight incubation. Well separated, discrete colonies were selected and restreaked into the corresponding agar plates to obtain pure colonies. Glycerol stock of cultures was maintained for future reference. Stringent measures like maintenance of asceptical condition and sub-culturing once every two weeks were practiced to avoid chances of mixed culture or contamination. MAC and EMB are selective medium for Gram- negative organisms that differentiates lactose fermenters (LF) from non-lactose fermenters (NLF). Biochemical characterizations for indole production, glucose fermentation, citrate utilization [11] were carried out for all the respective strains (in triplicates) after every sub-culture to study their profiles.

B. DNA Isolation

DNA isolation is a pre-requisite for NAATs especially PCR based techniques having several protocols and kits dedicated to the same with subtle difference in the components, steps and purity of DNA isolated [12]. Three trials were tried out for optimizing a quick and rapid method for DNA isolation. Pure *E.coli* cultures of 10 µl each were transferred to 2ml of nutrient broth in suspension tubes under aseptic conditions. These tubes were left

overnight undisturbed in shaker incubator. These suspensions were transferred into fresh 2ml eppendorff tube (T1-T3) following by centrifugation at 10,000 rpm for 5 min. The supernatant was discarded; pellets of T1 and T2 were resuspensed in the 100 μl of water. The suspension in T1 was vortexed vigorously for a minute to cause mechanical shearing of cell membrane whereas the suspension of the T2 was boiled for 5min in water bath (heat shock treatment). The pellet in T3 was resuspended in 100 μl of 3% KOH by continuous mixing by pipetting. Pipette tips with larger orifice were used to minimize shearing of DNA. All these methods were followed by a quick spin at 5,000 rpm for 2 min. Presence of DNA was analysed by agarose gel electrophoresis (AGE) [13] with 5 μl of supernatant from each tube. 0.8% agarose gel with 0.5μg ml^{-1} ethidium bromide (EtBr) was employed for this.

C. Molecular Assay of 16S rDNA Analysis

For performing PCR reaction mixture of 12.5 μl was prepared. The isolation step which gave intact DNA (minimal shearing) was chosen for the amplification reaction. The reaction soup comprised of 2μl of supernatant (DNA), 0.5 μM of forward primer-FP:16S (5'GTTAATACCTTTGCTCATTGA3') and reverse primer- RP: (5'AACAGGGTATCTAATCCTGTT3')[14], 1X of assay buffer with 1.5mM of $MgCl_2$, 800μM of dNTP mix and 0.5U/μl Taq polymerase (Kappa systems KK5004). The reactions were carried out in a thermal cycler (Bioer) with the program set for 25 cycles wherein each cyclic step comprised of denaturation (95^0C), annealing (60^0C) and extension (72^0C) for 30s each. The amplicon obtained were checked by gel electrophoresis on 2% agarose gel with 0.5μg/ml of EtBr at 5V/cm. Gels were viewed and photographed in Bioimaging system (Syngene). Standard genomic DNA of *E.coli* K12 (Merck) was used as the positive control and nuclease free water as negative control.

D.Multiple Sequence Alignment and Probe match

The complete genomic data of reference strains, *E.coli* K12 MG 1655 (NC_000913.2), *C.koseri* ATCC BAA-895 (NC_009792.1), *E.aerogenes* (NC_015663.1) and *K.pneumoniae subsp pneumoniae* MGH 78578 (NC_009648.1) were downloaded from National Center of Biotechnology Information (NCBI) in fasta format. The multiple locations of 16S ribosomal DNA (16S rDNA; *rrlS*) of each strain were noted and extracted using Perl stretch program. Multiple sequence alignment (MSA) of ribosomal segments within the species (Table I) was checked (Clustal W, EMBL). A representative of *rrl*S segment from each was chosen; MSA was executed to evaluate the extent of similarity among the four strains. Perl check program helped to validate positions and location of primers (both forward and reverse) within the targeted stretch. Perl programs were tailor made for the current study. The hybridization specificity of primers was cross-checked with Probe match (Ribosomal Database project: RDP).

III. Result And Discussion

All the four strains are LFs having almost similar morphology (pink colonies) on MAC with *K.pneumoniae* exhibiting mucoidal like colonies. *E.coli* was specifically distinguished from the rest on EMB, as they exhibited distinct green sheen colonies due to the metachromatic properties of the dyes eosin and methylene blue. Continuous sub-culturing showed phase variable colonies in EMB with *E.coli* exhibiting green sheen to coli-type colonies characteristic of the other strains. Phase variation are temporary reversible programmable events that lead a bacterium to switch between two or more morphology [15], [16].These diphasic expressions are problematic as these can result in fallacious assumptions leading to wrong medication. The reference strain of *E.coli* displayed positive Indole and Methyl red and negative Voges-Proskauer and Citrate test, whereas in *K.pneumoniae* and *E.aerogenes* these results are reversed. *C. freundii* gave positive Methyl red and Citrate test and negative Indole and Voges-Proskauer test. Biochemical results were seen to vary on sub-culturing with maximum false positives in Indole test and Methyl red tests. Interpretation of phenotypic tests often depends on the subjective judgment and the age of culture [17].

The analysis of DNA isolation methods showed promising results (Fig.1a), thus enabling an alternative yet rapid approach from kit methods. Shearing of the DNA was observed wherein vortexing was employed as the means of extraction (Fig.1a,lane1). Heat shock treatment (trial 2) caused complete denaturation of DNA showing only RNA smears on AGE. Prominent band of DNA was observed involving potassium hydroxide (KOH) treatment(Fig.1a,lane3).

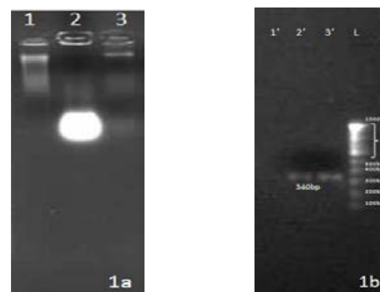

Fig.1a represents the DNA isolated from three trials using vortexing (1), heating(2) and KOH treatment (3). Fig.1b represents PCR products. Lane 2' shows amplification of DNA obatined from KOH method Lane 1' and 3' shows negative and positive control respectively. 100bp ladder was used to verify the amplicon size (L). The first gel is of 0.8% and the other is of 2% agarose

The choice of using of 3% KOH was made on the basis "sticky test" employed for confirming the Gram negative nature of bacterium [18].

PCR reaction targeting the 16S rDNA stretch gave a single band of 340bp for *E.coli* (Fig 1b). Unspecific bands were seen in the other strains especially *E.aerogenes* even after

pretreatment of the tubes, pipettes and tips under UV (20 min). Changing to fresh aliquots of DNA, PCR components and hand still gave inconclusive results. At majority of instances complete absence of bands were observed in other strains but at the same time less intense bands were shown in few occassions .These dubious results can stem due to incomplete genomic data at the time of primer designing. Genetic repeostories are continuous upgraded with whole and partial genome data from both cultured and uncultured microbial species. Annealing temperature, increased template concentration, mixed cultures and contamination can also lead to false positives [19]. Reference [14] reports coupling of 16S rDNA primer with dot-blot hydridization approach and real time PCR thus enhancing the specificty but at the same time is not cost-effective for routine tests.

MSA of the *rrl*S stretch of individual strains showed that the binding location of the primer is same in all. Analysis of forward primer across the sequences (Table II) showed 80% similarity match in *E.aerogenes* followed by *K.pneumoniae* and *C.freundii* with 70% and 65% complementarity. Sequence alignment of reverse primer showed specificity of 100% in all (Table III). The probe match. These unclassified cultures were of *Citrobacter*, *Enterobacter, Enterococcus*, *Klebsiella, Shigella* and *Staphylococcus* spp

TABLE I
PERL CHECKPRIMER DATA OF PRIMER LOCATION

Strains	*E*	*C*	*EA*	*K*
Primer location	224232—224572	4326335	4470481	4759440
	3940292--3940632	3645797	4364887	4004717
	4034015--4034355	2930735	4273814	
	4165143--4165483	2800156	4028251	
	4206631--4206971	2761938	3347018	

E: E.coli;C:C.koseri;EA:E.aerogenes;K:K.pneumoniae

Perl checkprimer.pl showed the FP and RP location within the genome of the respective strains. The locations were crosschecked positions of *rrl*S clusters and it was confirmed that the primer is specific to small ribosomal segment. Binding sites for both forward and reverse primer were recorded for *E.coli* but the program showed only binding sites for RP in the rest. The program gives a positive result only when a complete complementarily of the input sequence is found. MSA data (Table II & III) shows the extent of mismatches in the primer sequences among the strains

An ideal diagnostic tool should not only be sensitive but reproducible and authenticative. PCR technique coupled with sequencing can give better insight into the identity of the microbe but this again runs into trouble when blast search provide similar expected value (e-value) for

TABLE II
CLUSTAL W ANALYSIS SHOWING BINDING SPECIFICTY OF FORWARD PRIMER

Genomes	(FP) Forward Primer (on plus strand)	P	M	P%
E.coli	TCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAA**GTTAATACCTTTGCTCATTGA**	482	21	100%
C.koseri	TCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTG**GTTAATAACCGCAGCAATTGA**	482	13	62%
E.aerogenes	TCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCATTGTG**GTTAATAACCACAGTGATTGA**	484	16	76%
K.pneumoniae	TCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTGAG**GTTAATAACCTCATCGATTGA**	480	14	67%
	************* ********** ********* * ******* * *****			

P: position of the bp aligned; M: number matches of primer; P%;percentage match (M/primer length)

TABLE III
CLUSTAL W ANALYSIS SHOWING BINDING SPECIFICTY OF REVERSE PRIMER

Genomes	(RP) Reverse Primer (on plus strand)	P	M	P%
E.coli	**AACAGGATTAGATACCCTGGT**AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC	836	21	100%
C.koseri	**AACAGGATTAGATACCCTGGT**AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC	836	21	100%
E.aerogenes	**AACAGGATTAGATACCCTGGT**AGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTTCC	838	21	100%
K.pneumoniae	**AACAGGATTAGATACCCTGGT**AGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCC	834	21	100%
	***************************** ************** *********** **			

P: position of the bp aligned; M: number matches of primer; P%;percentage match (M/primer length)

mismatches were seen within the 8-16 bp stretch of 21bp long primer indicating less stable hybridization. The complete annealing specificity of RP can lead to chance amplification in samples; so mismatches of RP and FP in species specific primers is mandatory to avoid such occurence. Probe check (RDP) of the forward sequence confirmed such possibilities. Hits of 11301/1917495 was shown for bacterial domain, with e*nterobacteriales* order of class *γ-enterobacteriacea* showing 11240/48667 match. Out of the total 11049 uploaded data of unclassified_*enterobacteriaceae* 16S rDNA, 65 showed different strains. The simplest detour from such events can be a accomplished by a rigorous approach involving maximum utilization of software tools. Universal primers can be designed across bacterial species taking into account the restriction profile of the resultant target stretch. Instead of sequencing, the amplicons can be disgested to check for restriction pattern unique to that of a species or genus. Secondary structural analysis of ribosomal segments can verify the conservation of recognition sequences. Molecular assays are “gold standard” in comparison to the biochemical assay as they target the inherent gentic

material whereas the latter depenedent on the protein or enzyme produced. Any mutational events, intentional or of spontaneous nature can bring about frame shift in the reading frame leading to the absence or nonfunctional protein. Genotypic assays are thus superior in detection but complementing these with stringent standard operation procedure are important for validating results. The cumulative approach not only helps in understanding the niche, biochemical adapatation but aslo throws light into the evolutionary conservation of sequences.

REFERENCES

[1] M. Lorraine, G. Roger, “First, do no harm: the toll of unhealthy health care practices” in *American Enterprise Institute for Public Policy Research*,2007, No 13 accessed at < http://www.aei.org/article/health/medicaltechnology/pharmaceuticals/first-do-no-harm-outlook/>

[2] S.S Kanj, Z.A. Kanafani, N. Sidani, L. Alamuddin, N. Zahreddine, V.D. Rosenthal, “International nosocomial infection control consortium findings of device-associated infections rate in an intensive care unit of a Lebanese university hospital”, *J. Global. Infec. Diseases*, vol.4, no.1, pp.15-21,2012.

[3] V.D. Rosenthal *et al,* “International nosocomial infection control consortium report, data summary for 2002-2007”, *Am. J. Infect. Control*, vol.36, no.9, pp. 627-637, 2010.

[4] M. Rizvi, F. Khan, I. Shukla, A. Malik, Shaheen, “Rising prevalence of antimicrobial resistance in urinary tract infections during pregnancy: necessity for exploring newer treatment options”, *J. Lab. Physicians.,*vol.3, no.2,pp.98-103,2011.

[5] I. Okonko *et al*, “Detection of urinary tract infection (UTI) among pregnant women in Oluyoro Catholic Hospital, Ibadan, South-Western Nigeria”, *Malaysian J. Microbio*, vol 6, no.1, pp. 16-24, 2010.

[6] W.C. Winn, S.D. Allen, W.M. Janda, E.W. Koneman, P.C. Schreckenberger, G.W. Procop, G.I. Woods, “*Koneman's color atlas and textbook of diagnostic microbiology”*, Lippincott Willams and Wilkins. Baltimore, M.D, 2006.

[7] M. Manafi, B. Kremsmaier, “Comparative evaluation of different chromogenic fluorogenic media for detecting *Escherichia coli* O157:H7 in food”, *Int. J. Food. Microbiol*, vol.71,pp 257-262,2001.

[8] R. Arshad, S. Farooq, S.S. Ali, “Manipulation of different media and methods for cost effective characterization of *Escherichia coli* strains collected from different habitats”. *Pak. J. Bot*., vol.38, no.3, pp. 779-789, 2006.

[9] E.A. Mothershed, A.M. Whitney, “Nucleic acid-based methods for the detection of bacterial pathogens: Present and future considerations for the clinical laboratory”. *Clinica. Chimica. Acta*, vol.363, pp 206-220, 2006.

[10] A. Singh, R.V. Goering, S. Simjee, S.L. Foley and M.J. Zervos, “Application of Molecular Techniques to the Study of Hospital Infection”. *Clin. Microbiol. Rev,* vol. 19, no.3, pp. 512-530, 2006.

[11] M. Josephine, G. Morello, M. Helen, “*Laboratory Manual and Workbook in Microbiology: Applications to Patient Care”*, ed. 7th, McGraw-Hill, 2002, chapter 24, pp 153-158.

[12] K. Rantakokko-Jalava, K. Jalava, “ *Optimal DNA Isolation Method for Detection of Bacteria in Clinical Specimens by Broad-Range PCR” . J. Clin. Microbiol*., vol.40, no.11, pp. 4211-4217, 2002.

[13] J. Sambrook, E.F. Fritsch, T. Maniatis, “*Molecular cloning; a laboratory Manual”*, ed.2 , Cold Spring Harbour, NY: Cold Spring Harbour laboratory,1989

[14] E.Malinen, A.Kassinen, T.Rinttila¨, A.Palva, “Comparison of real-time PCR with SYBR Green I or 59-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria”, *Microbiology*, vol. 149, pp.269–277, 2003.

[15] M.V. Van der Woude, A.J. Bäumler, “*Phase and antigenic variation in bacteria*”. *Clin. Microbiol. Rev.*, vol.17, no.3, pp.581-611,2004.

[16] S.D. Singh ,J.R. Parvathi, “Epigenetic and non-epigenetic switch mechanisms in *Escherichia coli”. JPAM*., vol. 7, no. 1, 2013 (In press/ “Unpublished”).

[17] P.P. Bosshard, R. Zbinden, S. Abels. B. Boddinghaus, M. Altwegg and E.C. Bottger, “16S rRNA Gene Sequencing versus the API 20 NE System and the VITEK 2 ID-GNB Card for Identification of Non-fermenting Gram-Negative Bacteria in the Clinical Laboratory”, *J. Clin. Microbiol*, vol. 44, no.4, pp. 1359-1366,2006.

[18] E.M. Powers. “Efficacy of the Ryu Nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts”. *Appl. Env. Microbiol*, vol.61, no.10, pp.3756-3758, 1995.

[19] P. Apfalter , U. Reischl, M.R. Hammerschlag, “In-house nucleic acid amplification assays in research: How much quality control is needed before one can rely upon the results?”, *J. Clin. Microbiol*., vol. 43, no.12, pp. 5835-5841,2005.

Thermophysiological Responses and Heat Tolerance of Saudi Camel Breeds

Al-Haidary[1] A. A., E.M. Samara, A.B. Okab and K.A. Abdoun,

Abstract—The present study was conducted to investigate the variation in thermophysiological responses of four Saudi camel breeds. Sixteen dromedary native breeds (Almajaheem, Almaghatir, Alsafrah, Alzargeh) of 4 animals each were used in this study. Exposure of camel to hot summer (40o C) compared to spring (21o C) conditions resulted in variable breed-dependent thermophysiological responses. Where, the observed percentage increase in rectal temperature (Tr) and respiratory rate (RR) due to summer heat exposure were highest in Almaghatir and Alsafrah breeds, respectively and lowest in Alzargeh breed. The percentage increase in packed cell volume (PCV) was highest in Almajaheem and Alzargeh and lowest in Alsafrah and Almaghatir. Further, the obtained results indicate that coat color do not influence heat tolerance in camels. Nevertheless, further studies are needed to explore the role of breed variation in the structure of insulating coat and optical properties of hair on thermoregulation and heat tolerance of camels.

Keywords—: Breed, Camel, Heat tolerance, Thermoregulation.

.

I. INTRODUCTION

The one-humped camel (*Camelus dromedarius*) is a domestic animal of economic importance in some of the hotter, drier regions of the world, where it represents an essential source of meat and milk. The world camel population is regularly increasing with a yearly growth of 3.4% [1]. Saudi Arabia belongs to the countries with regular growth of camel population. The coat color represents the main criteria to name the breed in Saudi Arabia [1]. Accordingly many breeds are identified in Saudi Arabia including *Almajaheem* (black), *Maghatir* and *Alawark* (white), *Alhomor* (brown), *Alsafrah* (dark brown), *Alshaele* (grey to brown red), *Alawadi* (red to white), *Alsaheli* (red), *Alhadhana* (yellowish to red), *Asail* (yellow to brown) and *Alzargeh* (blue grey).

Heat tolerance superiority of an animal having a glossy, light coloured, i.e., a highly reflecting coat, over an animal having a dull, dark coat, can only be evaluated in the presence of solar radiation [2]. It has been reported that susceptibility to heat stress in individual animal is determined by many factors including previous exposure to heat stress, temperature, species, sex and condition score [3]. Energy exchange has been reported to be affected by skin and coat properties such as heat absorption, density, depth, diameter and colour [4]. Breeds colour has been indicated as an important influential of body temperature and heat tolerance ([5];[6], [7]. In the tropics, the pigmentation of the skin is essential in protecting deep tissues against excess solar radiation [8], where, light coats are the most desirable under such conditions [9];[10], [6] compared to dark coats which is known to gain more heat load from solar radiation. Skin characteristics (thickness, colour, sweat glands) and coat characteristics (angle to the skin surface, texture, intensity, diameter and length) determine the protective properties through affecting the routes of heat exchange (conduction, convection, radiation, evaporation) between the animal and the environment [11]. Genetic role have also been reported in cattle, where genes responsible for the expression of short coats in *Crioula* breeds have shown to determine part of their heat tolerance [12]-[14].

Heat tolerance and adaptation capacity to hot environments have been evaluated using physiological parameters including respiration, heart rate, body and skin temperatures, sweating rate, packed cell volume, potassium content in erythrocytes, individual heat tolerance coefficient, hormonal secretion and decreased rate of production [8], [15], [16],[17].

Despite the reported differences in the phenotypic coat color of camel breeds existing in Saudi Arabia [1]; there is no single report on heat tolerance variation between these breeds. Therefore, this study has been designed and conducted with the aim of exploring thermophysiological responses and heat tolerance of four Saudi camel breeds.

II. MATERIALS AND METHODS

This study was conducted during spring (21° C) and summer (40° C) seasons at Al-Kharj region, Kingdom of Saudi Arabia. Sixteen dromedary bull camels of native breeds (*Almajaheem, Maghatir, Alsafrah, Alzargeh*), 4 animals each, with mean body weight of 250±10.5 kg and 18 months of age were used in this study. Animals were housed as a group in a partially shaded pen with open yard, fed twice a day at 07.00 and 16.00 hours, and had free access to clean tap water.

Ambient temperature (Ta), relative humidity (RH), solar radiation and wind velocity were measured at 3 hours intervals for 2 successive days. Ambient temperature and RH were recorded using 2 data loggers (HOBO Pro Series data logger, Model H08-032-08, ONSET Co., Southern MA, USA) placed inside the pens. Thereafter, temperature-humidity index (THI) was calculated according to [18]. Solar radiation and wind velocity were recorded using black globe temperature and anemometer (Wilh. Lambrecht GmbH, Gottingen, Germany), respectively.

[1] Department of Animal Production, College of Food and Agriculture Sciences, King Saud University, 11451 Riyadh, Saudi Arabia, (96614678475) email: ahaidary@ksu.edu.sa