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Discovering Thoughts, Inventing Future

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Area Preference of GPS Tagged Re-Introduced Tigresses within their 100% MCPs in Sariska Tiger Reserve, India

By Gobind Sagar Bhardwaj, AJT Johnsingh, Gogul Selvi, Saket Agasti, Balaji Kari, Hemant Singh, Anand Kumar & GV Reddy

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Keywords: home range, area preference, tiger reserve, water holes, sweet spots.

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I. Introduction

Home range is an area where animal spends most of its time. It is normally traversed by an individual animal or group of animals during activities associated with feeding, resting, reproduction, shelter seeking and other factors important to an animal's survival (Burt 1943, Harestad & Bunnell 1979; Sanderson 1966). The size of a home range relates to body size (McNab 1963, Harestad & Bunnell 1979). Large mammals have larger home ranges than smaller ones, and carnivores generally have large home ranges than herbivores and omnivores of similar size. It has been argued that solitary female felids should maintain home ranges just large enough to contain enough prey to meet their energetic demands of reproduction, with exclusive home ranges expected only when resources are distributed evenly both spatially and temporally (Sandell 1989). Many studies have documented male felid home-range sizes much larger than expected based on energetic demands, suggesting that other factors such as maximizing breeding opportunities influence male home-range size and degree of exclusivity (Sandell 1989). Further, studies also demonstrated that, male spatial organization of tigers is because of area occupancy pattern by female tigers (Sandell 1989, MacDonald 1983). The larger home ranges of females of Amur tiger *Panthera tigris* altaica is associated with lack of sufficient prey and poor habitat quality (Goodrich et al. 2005, Goodrich et al. 2010, Miquelle et al. 1999) whereas in Indian sub-continent relatively small size of adult female home range is attributed to the spatially homogenous and high abundance of prey species (Sunquist 1981, Smith et al. 1987a, Smith 1993).

Tiger (*Panthera tigris* L.) is the largest of all felids and is considered as the most charismatic species in the field of wildlife conservation. It is found in diverse habitat types and shows remarkable tolerance to the variation in altitude, temperature and rainfall regimes (Sunquist et al. 1999). For the last few decades tiger has drawn attention from wildlife managers, biologists and conservationists. It occupies the top position in the food chain of the forest ecosystem. Therefore it occupies a prominent position in decision making of wildlife management across the globe, especially in tiger range countries. Decision- makers and managers often view the tiger number as the measure for the performance indicator of the managerial inputs in any landscape. However, many other indicators are often neglected due to tiger centric approach adopted by the stakeholders, especially in India, which is having the largest population of wild tigers across the globe.

Following the total extinction of tigers in Sariska tiger reserve (hereafter called STR) in 2004, reintroduction of tigers was done from Ranthambhore by translocating an initial population of five tigers (two males and three females), with a supplementation of two tigers (male and female) in every three years for a period of six years (Sankar et al. 2005). A total of 9 tigers from Ranthambhore have already been translocated to Sariska using different means of transport until the study period. Three male (ST1, ST4, ST6) and three female

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tigers (ST2, ST3, ST5) were brought from Ranthambhore. In the year 2012, ST2 delivered two cubs (ST7 and ST8). Later, two female orphan cubs, ST9 and ST10, were brought from Ranthambhore in 2013. While ST10 littered two cubs (ST11 and ST12) and ST2 delivered its second litter of two cubs (ST13 and ST14) in the year 2014. One cub (ST15) was reported to be borne from ST9 in the year 2016. At the beginning of the year 2018, two cubs were borne by ST14 and three cubs ST12. Again reporting of one cub from ST10 and three cubs from ST12 at the beginning of 2020 increased the tiger number in STR. With the reported mortality of three tigers (ST1, ST11 and ST4 and one missing (ST5) (possibly killed), the current population of tigers in STR is 20 (11 adults, 5 sub-adult and 4 cubs). While debating the area requirement for different individual tigers in human-dominated landscape of STR (e.g.1213.31 km^2), most of earlier studies suggested the role natural prey base (Sankar & Johnsingh 2002; Sankar et al. 2005; Sankar et al. 2010; Sankar et al. 2013) in determining the area occupancy of tigers. However, limited numbers of studies are available demonstrating the role of terrain, dense forest and availability of perennial water sources for determining the area preference of tigers in STR. With the increasing number of tigers in reserve having 26 villages, a national highway and more than 300 religious places, the available 1213 km^2 area of STR needs to be assessed. The present study was done to get answers to the following questions, (1) is there any role of dense forest in determining the area preference of tigers? (2) Is there any role of terrain and perennial water bodies in determining the area preference of tigers?

Three those were re-introduced tigresses from Ranthambhore, viz. ST9, ST10 and ST3 tigresses were collared with GPS Plus X transmitters on May 13, 2018; Nov16, 2018; and Nov 26, 2018. The ST3 was relocated from Ranthambhore in 2009 and the two orphan sibling females, ST9 and ST10, were relocated in 2013. The present study is based on the point locations as received from the satellite transmitters fitted in their collar and we attempted to answer the questions as cited above.

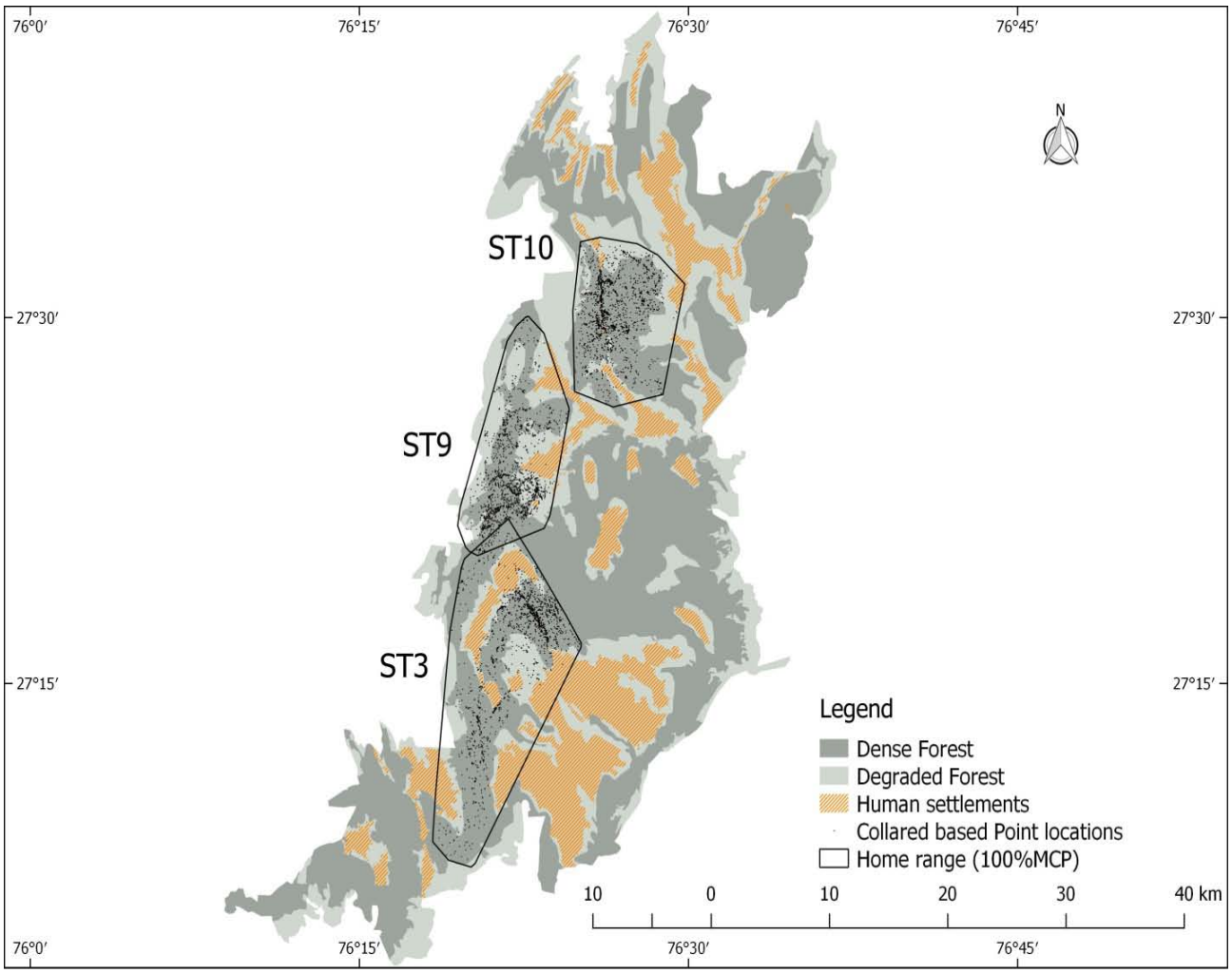

Fig. 1: Sariska Tiger Reserve showing area occupancies (MCP 100%) of ST3, ST9 and ST10 tigresses

II. Materials and Methods Area

The study area, the STR is situated in the Aravalli hill range and lies in the semi-arid part of Rajasthan (Rodgers and Panwar, 1988). The terrain is undulating to hilly and has numerous large to narrow valleys, two large plateaus—Kiraska and Kankwari and three large lakes, Silised, Mansarovar and Somasagar. The altitude of Sariska ranges from 240 to 777 m.

It is located in the Alwar district of the state of Rajasthan. After expansion its area was increased from 881 square kilometers to 1213.31 square kilometers due to the addition of buffer area (Fig 1) with some part of buffer (Jamwa Ramgarh Sanctuary) in the district of Jaipur. The vegetation of STR is tropical dry deciduous forests (Champion and Seth, 1968). The forest being scattered and sparse over a large area on various geological and soil formation and vary greatly in composition. *Anogeissus pendula* is dominant species in the undulating area and on the hills. *Boswellia serrata* and *Lannea coromandelica* grow on steep rocky areas. *Acacia catechu*, *Butea monosperma* and *Zizyphus mauritiana* are distributed in valleys. *Dendrocalamus strictus* is extremely limited in distribution and is present along the well-drained reaches of the streams and moist and colder part of the hills. Among bushes, *Grewia flavescence* and *Capparis sepiaria* form important components of understorey vegetation of the reserve. Apart from the tiger (*Panthera tigris* L.), other carnivores present in STR include common leopard (*Panthera pardus*), striped hyaena (*Hyaena hyaena*), jackal (*Canis aureus*), jungle cat (*Felis chaus*), common mongoose (*Herpestes edwardsi*), small Indian mongoose (*H. auropunctatus*), ruddy mongoose (*H. smithi*), palm civet (*Paradoxurus hermaphroditus*), small Indian civet (*Viverricula indica*) and ratel (*Mellivora camensis*). Till two decades caracal (*Caracal caracal*) were found in this reserve. Chital (*Axis axis*), sambar (*Rusa unicolor*), nilgai (*Boselaphus tragocamelus*) and wild pig (*Sus scrofa*) are the natural prey species for tigers found in STR. Other wild prey species found are Common langur (*Semnopethicus entellus*), Rhesus macaque (*Macaca mulatta*), Indian porcupine (*Hystrix indica*), rufous-tailed hare (*Lepus nigricollis ruficaudatus*), and Indian peafowl (*Pavo cristatus*). About 175 villages are situated in & around STR. Out of these, 29 (now 26 after relocation of three villages) are in Critical Tiger Habitat (CTH) and the rest 146 are outside the forest area. About 2254 families live in the core area, while about 12000 families live around the CTH (Shekhawat 2015), thus making this reserves a human-dominated landscape with immense anthropogenic pressures. Fig. 1 shows STR with a number of human habitations inside.

III. Methods

All of the tigers that were shifted from Ranthambhore were radio-collared. Tigers were immobilized with the mixture of Xylazine and Ketamine (500 mg + 400 mg, HBM) was used as 3.2-3.5 ml. A 250 kg container (length 5′ 11″, breadth 3′ 6.5″ and height 3′ 10″) was fabricated with non-slip wooden planks on the bottom and angle iron frames on sides and top for the transport of the animals (Sankar et al. 2010). Following the malfunctioning of collars of ST9, ST10 and ST3, these were replaced with Vertex plus VHF/GPS collar, VECTRONIC Aerospace Gmb using iridium satellite communication on May 13, 2018; Nov16, 2018; and Nov 26, 2018 respectively. Different GPSs of the collars for three tigresses were configured for different periods; accordingly, different number of coordinate locations was received for different tigresses. The time period for point locations were from May 13, 2018 to Nov 23, 2019 (ST9), Nov 16, 2018 to March 31, 2020 (ST10), Nov 26, 2018 to March 30, 2020 (ST3). The keyhole markup language (kml) file of these point locations was converted into shape files using open source GIS software (QGIS 2.18.19) and analysis was done with the help of software and MS Excel. The Minimum Convex Polygon (MCP) technique was used for home-range calculation (Mohr 1947, Anderson 1982 & Southwood 1996) and also for interpretation and comparison of home-range sizes. The use of MCPs was justified because of the sample size in the one-year study period and the temporally clustered nature of fixes that resulted in the autocorrelation of results (Swihart and Slade 1985). We estimated the age of each tiger based on their re-introduction record, known birth dates of young belonging to radio-collared mothers, evidence of having reproduced, and breeding behaviors noted after capture. The shape files for dense forests, degraded forests and agriculture settlements were generated using latest imageries from Google Earth viewed at 12675 feet. Spatial analysis was done using counts points in polygon in geometric and analysis tools of the vector using QGIS. The data for perennial source of water including the name of the water source, name of the beat, GPS coordinates, the status of water (present or absent) was collected monthly by the frontline staff for all beats from March 2018 to June 2019. The same data was sent to Field Director Office and consolidation was done in MS Excel.

IV. Observations

We obtained 2820, 2541 and 3613 point locations for tigresses ST3, ST9 and ST10 respectively for the period, as shown in table 1. The home ranges (100% MCP) computed for the period varies from 97.97 km^2 (ST10) to 189.82 (ST3). It was observed, as 104.98 km^2 for ST9. Fig 1 shows the home ranges three tigresses as shown as MCP (100%) in STR. These home ranges were observed to be considerably higher than other tigresses (eg.ST2, ST7, ST8) of STR during the period of study owing to the phylopatry behavior of

tigresses as ST7 and ST8 were borne to ST2. While demonstrating the percentage of time spent by tigresses in different habitats, we divided the habitat into three categories, *viz*. dense forest, degraded forest and human settlements/agriculture. The satellite received point locations of all tigresses were segregated from their respective 100% MCP home range using layer intersection and XY tools of Vector of QGIS 2.18.19. The proportional representation of point locations of tigresses in these three layers was taken as proportional time spent in these habitats. Maximum time was spent in dense forest (ST3=91%, ST9=72.2% & ST10=93.2%) followed by degraded forest (ST3=6.8%, ST9=27.6% & ST10=93.2%) and least was used with intensive anthropogenic activities (human habitations and agriculture) (ST3=1.6%, ST9=0.2% & ST10=0.5%). The computed area of different landscapes inside the individual home range (100% MCP polygons) is shown in table 1. Detailed observations for individual tigresses are described separately to understand the natural resource selection in relation to area preference.

Table 1

	ST3	ST9	ST10
Year of relocation	2009	2013	2013
Age of tigress in years	14	10	10
Date of putting GPS collar (year 2018)	23 Nov	13 May	16 Nov
Period of data in number of days	492	559	502
Point locations for tigresses	2820	2541	3613
Point locations in dense forest	2585	1835	3369
Point Location in degraded forest	191	702	225
Point location in human habitations and agriculture	44	4	19
Home range of individual tigress MCP(100%) in Km^2	189.82	104.98	97.97
Dense forest in inside home range MCP(100%) in Km^2	109.9	56.13	68.01
Degraded forest inside home range MCP(100%) in Km^2	79.92	48.85	29.96
Percent of dense forest in home range in Km^2	57.9	53.5	69.4

Tigress ST3

One of the siblings of three female cubs of fourth litter from T16 tigress of Ranthambhore born in 2006 was relocated to STR in 2009 as a part of the re-introduction of tigers. Her predecessor tigress ST2 had already occupied Sariska-Kachida valley, an important area or sweet spot of STR with plenty of prey base. While occupying southern portion of STR, 70.37% of her reported kills (based on individual tiger monitoring) were those of livestock, including buffaloes and cows between 2016 to 2018 (Bhardwaj et al. 2020). Although the percent of dense forest inside the home range of ST3 was 57.9%, the tigress was observed to spent 91% of her time in dense cover while rest of the time in degraded areas, including human-dominated landscapes. While attempting to demonstrate the temporal segregation of time period in degraded forests and human habitations including agriculture fields, ST3 was observed wandering mostly during night hours (viz. 62.30% in degraded areas and 69.91% in human-dominated landscapes) among the total observations.

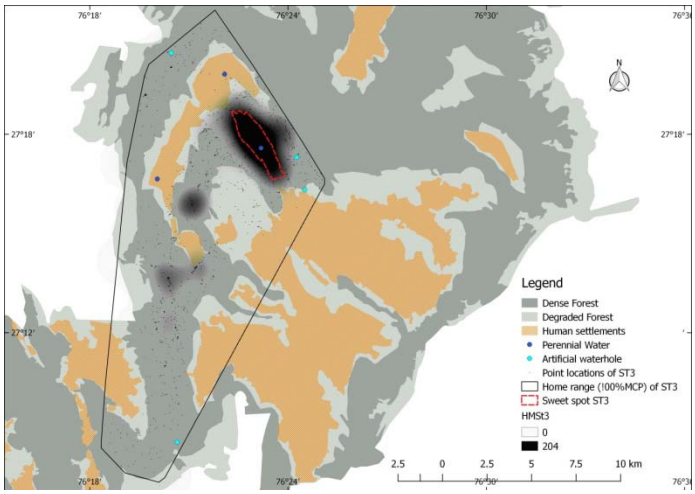

Fig. 2: Home range (MCP100%) of ST3 tigress showing with heat map, sweet spot, and water points in different forest types. Whereas AWHs is artificial water holes and NWS is natural water source

Based on the data analysis of the water tracking report from March 2018 to June 2019, sixty natural perennial water holes were identified in STR. These perennial sources were observed to have water for the whole of the year. The perennial water sources falling in home range of tigress ST3 along with different landscapes in the MCP100% polygons are shown in figure 2.

Among all natural water source (NWS) as shown in the polygon, only one water point is in dense forest and the rest of the points (NWSs) are located in human dominated landscapes including habitations and agriculture fields.

Tigress ST9

ST9, with an approximate age of 10 years, is one of the two orphan cubs (another sibling is ST10) those were translocated from Ranthambhore tiger reserve in the year 2013. She reportedly gave birth to one male cub, ST15 in, 2015, who has established its territory in the southern portion of STR. Based on point locations (n=2541 of all 559 days), the observed home range (MCP100%) of ST9 is 104.98 km^2. Almost half of the area (56.13%) of its home range is having dense forest where it was observed to spend the third quarter of her time (72.2%) and rest in the degraded forest (27.6%) and human-dominated landscapes (0.2%). While comparing with ST3 tigress, it was observed that comparatively considerable period was spent in degraded areas of its polygon by ST9.

The tigress was observed visiting equally during the day (52%) and night hours (48%) in degraded areas.

Five perennial water points were observed to be located in the southern half portion of the home range (MCP100%) of ST9. Even the artificial waterholes which were being filled for the whole of the year also occupy the southern half of the home range polygon. The observation of location congregations of tigress ST9 in the southern half of occupancy polygon suggests affinity of the tigress to the proximity of water holes, especially perennial sources.

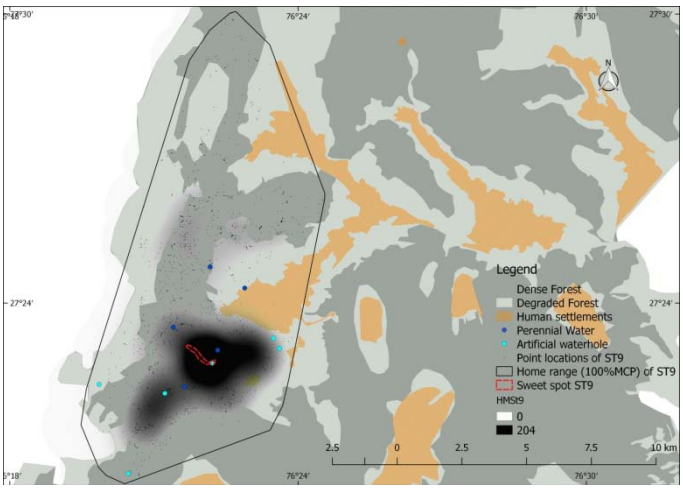

Fig. 3: Home range (MCP100%) of ST9 tigress showing with heat map, sweet spot, and water points in different forest types. Whereas AWHs is artificial water holes and NWS is natural water source

Tigress ST10

ST10, with an approximate age of 10 years, a sibling of ST9, that was translocated from Ranthambhore tiger reserve in the year 2013. The computed area of the home range (MCP100%) for ST10 was observed as 98 km^2, which is almost half of the ST3 tigress. Two-third of its area (69.4%) in the home range of ST10 is of dense forest and the rest (30.6%) is either the degraded area or with human-dominated landscapes with human habitations and agriculture. Among all received point locations (n=3613) for ST10 tigress, 93.2% were observed in the dense forest, although the proportion of rest of the degraded and human-dominated landscape was roughly one-third (30.6%).

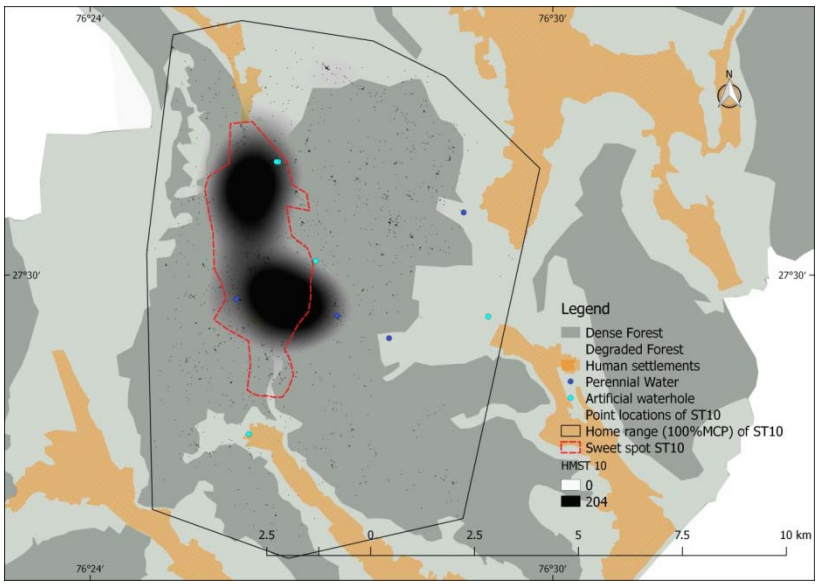

Fig. 4: Home range (MCP100%) of ST10 tigress showing with heat map, sweet spot, and water points in different forest types. Whereas AWHs is artificial water holes and NWS is natural water source

Four perennial water sources with a permanent source of water for the whole of the year are located in the home range of ST10. Among these, two are located in the center of the polygon in dense forest where the congregation of point location of ST10 tigress was observed maximum and two water points in degraded forest area showing less congregation (fig 4).

While attempting to map the sweet spots of all these three tigresses in their respective home ranges, maximum preference was given by individual tigresses in the valleys and gorge areas. Fig 2, 3 and 4 show the heat maps generated from point location spatial data using QGIS. An area of 4.13 km^2 locally called Baghani was observed to be intensively used by tigress ST3 with 683 point locations, which are approximately 11 times more than those in MCP100% for total home range. While analyzing the terrain of this polygon, it was observed to be a narrow valley with perennial water source in the area. Similarly, the observation of maximum utilization of valley by ST9 tigress with 243 point locations in just 0.17 km^2 which is approximate 59 times more than rest of the area of polygon, demonstrates the tigress's preference for the valley. The computed maximum congregation of point locations (n=248) of tigress ST10 in just 10.9 km^2 is again a valley area) with thick vegetation dominated by *Butea monosperma* species.

V. Discussion

Earlier Sunquist (1981) demonstrated that both felids, tiger and leopard, are territorial and wide-ranging. Still the effective size of the territory is the function of density and biomass of larger prey species in their habitat. In STR too, past studies highlighting the role of natural prey base in determining the area occupancy of wild tigers have already been demonstrated in tiger range countries (Sankar & Johnsingh 2002; Sankar et al. 2005; Sankar et al. 2010; Sankar et al. 2013). Above studies demonstrate the role of natural prey base in determining the area occupancy of tigers. In human-dominated landscapes where the proportion of natural prey base is far below than livestock available in proximity to a predator as evident from a study done in the same landscape (Bhardwaj et al. 2020), the role of other factors like human disturbance, natural cover, availability of water or terrain of the area can be viewed as determining factors for area preference for tigers. Based on reported kills by tigers in STR (between 2016 and 2018), the observation of a maximum number of kills of livestock (77%) followed by Sambar *Rusa unicolor* (13.6%), chital *Axis axis* (3.6%), blue bull *Boselaphus tragocamelus* (2.4%) and wild pig *Sus scrofa* (0.95%) demonstrates the less dependence on natural prey base in determining the area preference

tigers (Bhardwaj et al. 2020). When the number of unaccompanied livestock in an area is more than wild prey, naturally, tigers will go for livestock. Apart from the role of prey, here we attempted to highlight the significance of dense forest (cover), terrain and perennial water sources in area preference of tigers within their home ranges. Almost all of the three tigresses showed the preferences for the dense forest with the maximum percentage by ST3 (91% of all point locations for 58% dense forest within MCP100% of ST3) and ST10 (93.2% of all point locations for 69.4% dense forest within MCP100% of ST10). Although ST9 also preferred for the dense forest but relatively less (72.2% of all point locations for 53.5% dense forest within MCP100% of ST9) as compared to ST3 and ST10. It can be explained by the presence of several perennial water holes as well as artificial waterholes in the degraded area too especially in southern portion of the home range of the tigress. These waterholes also explain the skewed distribution of location points of ST9 in the lower half of her home range.

Spending most of the time in dense forest (91% point locations), the biggest congregation of point locations of ST3 was observed to be located in the north-portion of its home range (Fig 2). The reason for this high congregation can be attributed to the presence of a perennial natural source (no.1). Although there are three other perennial natural sources (NWS) however these are located in degraded areas and near human habitations, thus tigress avoiding these locations. Similarly, the observation of the largest congregation of point locations near two perennial water sources in dense forest within the home range of ST10 tigress shows its preference for perennial water sources in the dense forest only. Largest congregations of point locations of all these tigresses in valleys also demonstrate the importance of terrain, especially deep gorges and valleys as area preference by mega carnivores. Thus, valleys with dense vegetation and availability of perennial water sources throughout the year in the semi-arid landscape of Aravallis are the determining factors for area preference for tigers when natural prey base is not only a limiting factor. The skewed location of natural perennial water holes in home ranges of individual tigresses further invites the managerial interventions for selecting sites for artificial water holes in a continuous pattern inside the home range polygons. The preference of tigresses for dense forest again highlights the urgent need to protect the degraded forest areas of the STR from anthropogenic pressures to enhance the vegetative cover. This can be done only through the voluntary relocation of villages from critical tiger habitat and strengthening of enforcement regime of Sariska administration through immediate filling up of the vacant posts of forest guards, creation of Sariska Tiger Protection Force (STPF) and reviewing the strength and number of existing frontline staff (Bhardwaj 2018). As the tiger number is increasing through strict tiger monitoring in reserve, we also recommend that such sweet spots to be identified for all tigresses and necessary managerial efforts are to be made to make these areas as totally free from any anthropogenic activities. While the process of voluntary relocation of villages from STR is certainly going to take some time, the protection of at least these high congregation areas called sweet spots of the tiger will act as escape refuge for these tigers in the human-dominated tiger reserve.

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Transcriptomic Profiling of Pseudomonas Putida (NBAII RPF9) Exposed to Heat and Salt Shock

By Ashwitha K & Rangeshwaran R

Abstract- *Pseudomonas* spp. is one of the widely studied bacterium for its versatility to be used for agricultural purposes as bioagent, biostimulant, biofertilizers or biopesticides. However, the dynamics of its efficacy varied from lab to field. The advanced biocontrol technologies have enabled better understanding of adaptability of selected *Pseudomonas* for field applications. In our study, we subjected *P.putida* (NBAII RPF9) strain to heat shock and salt shock followed by microarray analysis of the gene expression under both stresses when induced independently. Few genes were expressed under both conditions whereas some of them were newly reported stress tolerance genes. The quantification of selected stress tolerance genes revealed the level of fold change of these genes under stress and its role in conferring mitigation of heat and salt shock to *P.putida* (NBAII RPF 9). These techniques can be considered as major criterion in selection of a potential bacterial strain for agricultural applications.

Keywords: *pseudomonas putida, heat shock, salt shock, microarray, RT PCR, chaperones, stress genes, osmoprotectants, GroES, DnaK.*

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Ashwitha K[α] & Rangeshwaran R[σ]

Abstract- ***Pseudomonas*** **spp. is one of the widely studied bacterium for its versatility to be used for agricultural purposes as bioagent, biostimulant, biofertilizers or biopesticides. However, the dynamics of its efficacy varied from lab to field. The advanced biocontrol technologies have enabled better understanding of adaptability of selected *Pseudomonas* for field applications. In our study, we subjected *P.putida* (NBAII RPF9) strain to heat shock and salt shock followed by microarray analysis of the gene expression under both stresses when induced independently. Few genes were expressed under both conditions whereas some of them were newly reported stress tolerance genes. The quantification of selected stress tolerance genes revealed the level of fold change of these genes under stress and its role in conferring mitigation of heat and salt shock to *P.putida* (NBAII RPF 9). These techniques can be considered as major criterion in selection of a potential bacterial strain for agricultural applications.**

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I. Introduction

The complex soil ecosystems are under constant exposure to multitudinous changes due to several biotic and abiotic factors. This hugely impacts its microflora in terms of their survival and existence. Abiotic stress factors like temperature, salinity, water and nutrition deprivation stress are uncontrolled and bring about manifold changes in survivability of micro organisms which are regulated by expression of genes, leading to series of events which assist in its adaptation through temporary slowing, physiological changes and release of protective compounds (Weller, 1998; Swiecelo *et al*., 2013). Bacterial species adapt themselves by altering their physiological and molecular processes and counter balancing their existence under unfavorable conditions. Therefore, promising bacteria which have biocontrol ability and tolerance to extreme environmental conditions can be used as a bio agent for plant health. *Pseudomonas* being one of the major ubiquitous soil microbes also faces these unprecedented circumstances in the soil. There are countless documentation of survival and fitness of different *Pseudomonas* strains recorded under different environmental stresses. *Pseudomonas* mitigates the stress by producing stress sigma factors and molecular chaperones unlike in cold shock where metabolic changes are induced. These stresses influence the membrane fluidity, transcriptional and translational defects in cells (Noor *et al*., 2019). The expression of heat stress related genes like heat shock proteins (Hsp), membrane regulatory proteins, ATP binding proteins, expression of osmoprotectant genes are some of the mechanisms by which *Pseudomonas* have shown to survive through different stress conditions (Ritcher *et al*., 2010). A general stress Sigma factors RpoS and RpoE are one among involved in survival of *Pseudomonas* strains under stress. During salt shock responses, *P.* aeruginosa producing spectrum of osmoprotectant which enables its survival under osmotic stress (Novik *et al*., 2015). Some of the research shows that stress exposed bacteria shift to viable but non culturable states which serves as the nutrient for the surviving population (Arana *et al*., 2010). *Pseudomonas* sp. enter into viable but not culturable during adaptation to environmental changes like oxygen limitation, high NaCl concentrations and high temperatures. The VNBC state cannot be considered as a physiological indication of survival in adverse conditions. The VNBC state of the *P. putida* might become part of complex functioning of the ecosystem but its actual role and important needs to be ascertained. The ability to survive under different stress deferred with *Pseudomonas* species. The abiotic stress impacts are well documented with *P. aeruginosa* strains with respect to medical applications. Scanty reports are generated on understanding the survival mechanisms of agriculturally important *Pseudomonas*. Some of the studies have proved that *Pseudomonas* spp shows remarkable effects in plants survival which is exposed to environmental stresses. P *.putida* subclade has alleviated the impacts of saline stress in plants which were inoculated with the strain. This proves that *P. putida* can be considered a potential strain in plant applications as well. The inoculation of *P. Putida* even enhances plant based stress responses in plants (Chu *et al*., 2019). In our independent studies we have established that *P. putida* enhances plant growth under high salinity (Ashwitha *et al*., 2018; Rangeshwaran *et al*., 2013). Currently it is important to generate data and understand the mechanisms a potential *Pseudomonas* strain adopts to survive under extreme environmental changes. In our present study we demonstrated the

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mechanism adopted by *P. putida* (NBAII RPF9) when subjected to heat shock and salt shock under laboratory culture conditions. Transcriptomic profiling of stress exposed *P.putida* (NBAII RPF9) using Microarray technique and few of the selected stress related genes were quantified using qRTPCR.

II. Material and Methods

a) Bacterial strain preparation

P. putida (NBAII RPF 9) was used as a model system in the study. Initial inoculum was prepared by inoculating fresh culture of the bacterium to 10 ml of sterile LB liquid medium taken in 30 ml glass screw capped vial. The culture was grown overnight at 28 ± 2 °C under constant shaking. The overnight culture was used to inoculate two sets of 100 ml sterile LB liquid medium each taken in 250 ml Erlenmeyer flasks. The inoculated broth was incubated overnight at 28 ± 2 °C under constant shaking. The stress was induced in the following method.

Saline shock: At mid log-phase of growth, solid NaCl was added to one of the flasks to obtain 1 M as final concentration in the medium and maintained for 1 h to subject it to saline shock.

Heat shock: P. putida (NBAII RPF9) was grown in LB broth at 28 ± 2 °C overnight and cells were harvested by centrifuging at 10,000 rpm for 5 min. The pellets were washed twice with 50 mM phosphate buffer, pH 7.0 and resuspended in 25 ml LB broth. Heat shock was induced by subjecting the culture to 45 °C for 20 min in a water bath. Post treatment, the cultures were centrifuged at 10,000 rpm for 10 min. The cell pellets were frozen until use.

b) RNA isolation and purification

RNA isolation was carried out using Qiagen's RNeasy minikit Cat #74104 using manufacturer's instructions. The bacterial cell pellets were transferred to two ml centrifuge tubes suspended in 600 µl of Buffer RLT and lysed using acid washed glass beads with high speed agitation (1000 x g for 5 min) to release the RNA. After a mechanical disruption method, supernatant was decanted. The method was repeated twice for complete disruption of cells. The lysate was left undisturbed for the beads to settle. The lysates were transferred to new microcentrifuge tubes and centrifuged for 2 min at 1000 x g. After centrifugation, 350 µl of supernatant was transferred to new microcentrifuge tubes. To this equal volume of 70% ethanol was added and mixed by pipetting method. 750 µl of total sample was transferred to RNeasy spin column placed in 2ml of collection tube. The lid was closed and the centrifuged for 15s at 10,000 rpm. The flow through collected at the bottom of the collection tube was discarded. 700 µl of Buffer RW1 was added to the RNeasy column. The tubes were again centrifuged for 15s at 10,000 rpm. The flow through was discarded. 500 µl of buffer RPE was added to the tubes and centrifuged again 15s at 10,000 rpm and flow through was discarded. The step was repeated again with centrifugation for 2 min at 10,000 rpm to wash the spin column membrane. The RN easy spin column was placed in a new collection tube of 1.5 ml volume. 50 µl of RNase free water was added to the spin column membrane and centrifuged at 10,000 rpm for 1 min to elute the RNA. The method was repeated once again for high RNA yield recovery. The purified RNA was stored in -20°C until used. RNA quality and purity was determined using Nanodrop spectrophotometer and bioanalyser. 10 µl of RNA sample was mixed with 490 µl of 10mM Tris Cl, pH (7.0). 500 µl of Tris buffer was considered as control. The absorbance of the sample was determined at 260 nm.

c) Hybridization and processing of microarrays

Hybridization was carried out in custom designed slides from Agilent technologies using eArray of 8x15K array AMADID 033142. Total RNA (200 ng) was mixed with 1.2 µl of T7 promoter primer in 1.5 ml microcentrifuge tubes. 2 µl of spike mix was added (Agilent One colour quick amp kit) to this mixture and made up to 11.5 µl using nuclease free water. Primer and the template were denatured by incubating the reaction at 65 degrees in a shaking water bath for 10 minutes. Later, the reaction was placed on ice for 5 min. Soon later, the 8 µl of cDNA master mix was added and again placed on ice. The contents were mixed using a pipette and later incubated at 40°C in a shaking water bath for 2 hours. The samples were further maintained at 65 degrees for 15 min in the water bath. Samples were placed on ice intermediately during sample preparation. 60 µl of transcription master mix was added to the above mixture. Incubate this mixture at 40°C for 2 hours.

d) cRNA purification

The total volume of cRNA was made to 100 µl with nuclease free water. The samples were purified using a column method. The cRNA samples were quantified directly using Nanodrop ND-1000 UV-VIS Spectrophotometer. One µl of cRNA elute sample was used for estimation directly. 1 µl of nuclease free water was used as blank. The concentration of cRNA (ng/µl) was determined using the following formula;

Concentration of cRNA * 30 µl (elution volume)/1000 = µg of cRNA.

The hybridization was carried out using Agilent gene expression Hybridization kit. The hybridised samples were dispensed on Agilent SureHyb provided with the Agilent Microarray Hybridization Chamber Kit (G2534A). The hybridization was carried out at 65 °C for 17 hours.

e) Data analysis

The microarray data was imported in excel format containing sample, array details and summary of

differentially regulated genes. The data were segregated as genes showing >1 (up regulated) and genes showing <1 (down regulated) along with annotations. The entire data set was submitted to NCBI GEO, a public database for functional genomics data repository supporting MIAME-compliant data submissions. The array data submissions formatted into Metadata worksheet according to GEO submission format for Agilent one colour experiment. The array data was accepted after scrutiny and provided with GEO accession number.

f) Real time quantitative Reverse transcriptase PCR analysis

Real time PCR was used to validate the results obtained from Microarray studies. A total of 25 genes were selected from the microarray gene list. The study was carried out as follows.

i. *Primer design*

A total of 25 stress related genes were selected for the study from a microarray database. Primers were designed and synthesized by Chromous Biotech, Bangalore for quantitative analysis of these genes when differentially expressed under stress conditions. *P. putida* gyrase gene was considered as housekeeping genes. mRNA expression level analysis of differentially expressed genes of P.putida (NBAII RPF 9) under stress was done based on relative quantification of the housekeeping gene.

ii. *RT PCR analysis*

The bacterial sample was prepared as mentioned above. RNA was isolated from culture pellets obtained by method followed in 3.6.3.1. First strand cDNA was synthesised by the following reaction containing 2 ng of RNA as template to which 1μl of oligo dT primer was added in a micro centrifuge tube. This mixture was mixed and the tube was heated to 70 °C for 5 minutes. The tube was immediately placed on ice. To this mixture, 5 μl of MmuLV buffer, 6 μl of dNTP mix (10mM), 1μl of MmuLV reverse transcriptase enzyme and the entire mixture was made up to 25 μl of reaction mixture with nuclease free water. The tubes were gently mixed and incubated 60 min at 42 °C. The reaction was terminated by heat inactivation and was done by incubation at 75 °C for 5 minutes. RT PCR was performed using Qiagen SYBR PCR kit performed according to manufacturer's protocols. Qiagen SYBR PCR master mix stored in -20 °C was thawed before use. Reaction mixture was prepared containing 2 μl of template, 2 μl each of forward and reverse primers (0.3 μM), 25 μl of 2X PCR SYBR green ready mix and this mixture was made up to 50 μl using RNAse free water. The master mix was mixed thoroughly. The PCR reaction was run on ABI Step-one Real Time PCR machine. All samples were amplified in triplicate from the same RNA preparation and the mean value was considered. The following amplification program was run. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (CT). CT values from the ABI Step One Plus Real Time PCR (Applied Biosystems, USA) were analysed.

III. Results

The bacterial samples subjected to different stress were analysed for the gene expression patterns. The microarray analysis provided data on genetic expression *P. putida* (NBAII RPF 9) under heat and salt shock conditions. A total of 5338 genes were expressed by *P. putida* (NBAII RPF 9). The Microarray slides were hybridised with reference genes from *P. putida* KT2440, a reference strain. The expression of each gene was normalised to its expression under normal growth conditions. We used the cut off value 2 for fold change in expression.

a) Transcriptome profile of P. putida (NBAII RPF 9) subjected to heat shock conditions

The microarray data revealed that 692 genes were up regulated and 542 genes were down regulated under heat shock conditions when induced to *P. putida* (NBAII RPF 9) (Fig.1). Microarray results affirmed the detailed list of differentially expressed genes which played a major role in transmitting stress response in *P. putida*. Among the 692 genes up regulated, 60.9% of the genes were absent under non stressed conditions and were only expressed under heat shock. In addition, 19% of the down regulated genes were seen to be absent under heat shock conditions. Rest of genes were present under both non stressed and stressed conditions whereas differential expression was observed in both up regulated and down regulated genes. We observed few genes which were not reported earlier for its stress response in *Pseudomonas* sp. Most of the genes over expressed above 5 fold included heat shock related genes coding DnaK and genes regulating transport activity. CinA domain protein showed -11.89 fold decreases under heat shock, a natural competence proteins found in *Thermus thermophilus*. Their actual mechanism of stress tolerance remained unexplained (Table 1 and Table 2). However, we were able to also screen many genes which were involved in transport activity, DNA repair, ATP binding and transcription related genes.

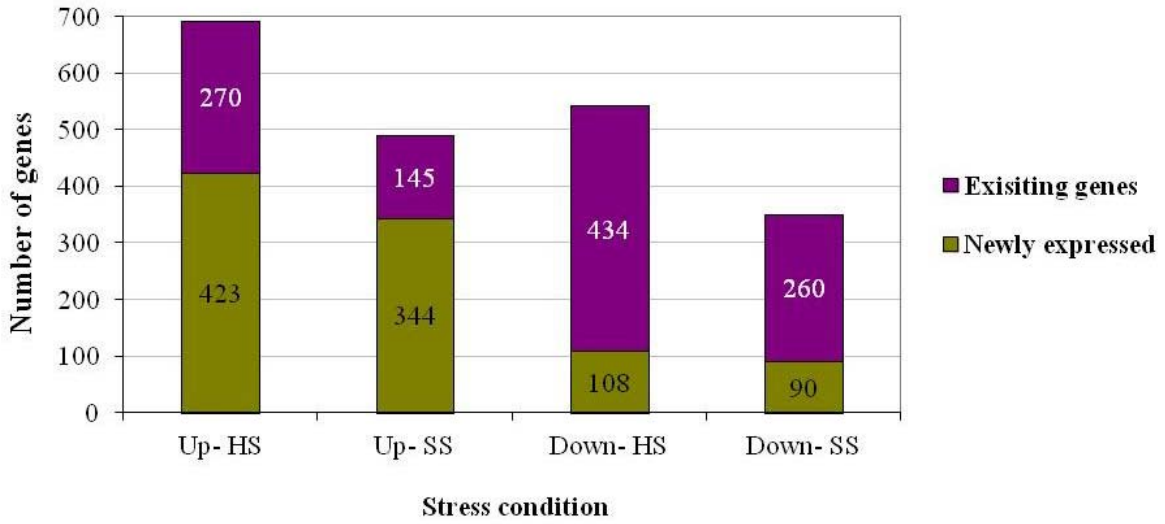

Fig. 1: Total number of differentially expressed genes under different stress conditions; genes up regulated under heat shock (Up-HS), genes up regulated under salt shock (Up-SS), genes down regulated under Heat shock (Down-HS) and genes down regulated under salt shock (Down-SS).

Table 1: Most relevant gene expression found up regulated under heat shock in P. putida (RPF 9)

ID	Name	Gene ontology	Heat shock
			Fold change
PP3044 PP_3044	Phage portal protein, lambda family	DNA binding; viral capsid	7.80
PP3371 PP_3371	Sensor histidine kinase	ATP binding; integral to membrane; peptidyl-histidine phosphorylation; two-component sensor activity	6.75
pcaD PP1380 PP_1380	3-oxoadipate enol-lactone hydrolase	3-oxoadipate enol-lactonase activity; catechol catabolic process	6.02
PP5376 PP_5376	Putative uncharacterized protein		5.96
glmM PP_4716	Phosphoglucosamine mutase (EC 5.4.2.10)	carbohydrate metabolic process; magnesium ion binding; phosphoglucosamine mutase activity	5.82
PP3617 PP_3617	Putative uncharacterized protein		5.46
PP3613 PP_3613	L-sorbosone dehydrogenase	catalytic activity	5.30
PP3808 PP_3808	Putative uncharacterized protein		5.28
PP4422 PP_4422	Succinate-semialdehyde dehydrogenase, putative	oxidoreductase activity	5.14
PP0709 PP_0709	Transporter, NCS1 nucleoside transporter family	membrane; nucleobase transmembrane transporter activity	5.13
gntP PP3417 PP_3417	Gluconate transporter	gluconate transmembrane transporter activity; membrane	5.11
dnaK PP_4727	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat shock protein 70)	ATP binding; protein folding; response to stress; unfolded protein binding	5.10
tyrB-2 PP3590 PP_3590	Aromatic-amino-acid aminotransferase	biosynthetic process; cellular amino acid metabolic process; pyridoxal phosphate binding; transaminase activity	5.08
hslU PP_5001	ATP-dependent protease ATPase subunit HslU (Unfoldase HslU)	ATP binding; ATPase activity; HslUV protease complex; peptidase activity, acting on L-amino acid peptides	5.02

Table 2: Most relevant gene expression found down regulated under heat shock in P. putida (RPF 9)

ID	Name	Gene Ontology	Heat shock Fold change
PP1628 PP_1628	CinA domain protein		-11.89
PP2297 PP_2297	Integrative genetic element Ppu40, integrase	DNA binding; DNA integration; DNA recombination	-10.93
PP0242 PP_0242	Transcriptional regulator, TetR family	DNA binding; regulation of transcription, DNA-dependent; transcription, DNA-dependent	-5.97
PP3689 PP_3689	Serine/threonine protein phosphatase, putative	hydrolase activity	-5.76
PP1195 PP_1195	Putative uncharacterized protein		-5.45
rpoA PP_0479	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha) (EC 2.7.7.6) (RNA polymerase subunit alpha) (Transcriptase subunit alpha)	DNA binding; DNA-directed RNA polymerase activity; protein dimerization activity	-5.26
PP1130 PP_1130	Putative uncharacterized protein		-5.02
PP1146 PP_1146	Putative uncharacterized protein		-4.83
cyoE2 cyoE-2 PP_0816	Protoheme IX farnesyltransferase 2 (EC 2.5.1.-) (Heme B farnesyltransferase 2) (Heme O synthase 2)	heme O biosynthetic process; integral to membrane; plasma membrane; protoheme IX farnesyltransferase activity	-4.58
PP3451 PP_3451	Putative uncharacterized protein		-4.50
PP4623 PP_4623	Putative uncharacterized protein		-4.35
PP2896 PP_2896	Putative uncharacterized protein		-4.24
PP3007 PP_3007	Putative uncharacterized protein		-4.08
PP2516 PP_2516	Transcriptional regulator, LysR family	DNA binding; regulation of transcription, DNA-dependent; sequence-specific DNA binding transcription factor activity	-3.81

b) Transcriptome profile of P. putida (NBAII RPF9) subjected to salt shock conditions

In case of induced salt shock with 1M NaCl, we observed up regulation of 489 genes and down regulation of 350 genes (Fig.1). 70.3% of the genes were newly up regulated whereas 29.6% of up regulated genes were present under both non stressed and saline shock conditions. Most relevant genes expressed with more than 2 fold change under stress are documented in Table 3 and Table 4. The transcriptome profile exhibited genes involved majorly in membrane transport, production of osmoprotective compounds and transcriptional regulators. We observed more than 5 fold expression of outer membrane proteins like opr1 and periplasmic polyamine-binding protein. Thirteen major stress related proteins involved in stress response were selected for RT PCR validation expressed under salt shock and having known functions in stress tolerance. After analysing the Microarray data, genes responsible for stress tolerance were selected for validation using RT PCR studies. The full database was submitted to the GEO database. The NCBI GEO accession for the microarray profile is mentioned below

1. *GSE103282* – Investigation on gene expression of *Pseudomonas* putida (NBAII RPF 9) under abiotic stress [heat].
2. *GSE103283* – Investigation on gene expression of *Pseudomonas* putida (NBAII RPF 9) under abiotic stress [salt].

Table 3: Most relevant gene expression found up regulated under salt shock in P. putida (RPF 9)

ID	Name	Gene Ontology	salt shock
			Fold change
PP0312 PP_0312	Electron transfer flavoprotein, alpha subunit	electron carrier activity; flavin adenine dinucleotide binding	6.05
PP2021 PP_2021	Putative uncharacterized protein		6.01
PP2041 PP_2041	Transcriptional regulator, LysR family	DNA binding; regulation of transcription, DNA-dependent; sequence-specific DNA binding transcription factor activity	5.87
pqqD2 PP_2681	Coenzyme PQQ synthesis protein D 2 (Pyrroloquinoline quinone biosynthesis protein D 2)	pyrroloquinoline quinone biosynthetic process; quinone binding	5.70
PP3211 PP_3211	ABC transporter, ATP-binding protein	ATP binding; ATPase activity	5.58
PP0955 PP_0955	Putative uncharacterized protein		5.17
PP3236 PP_3236	Lipoprotein OprI, putative		5.09
PP3147 PP_3147	Periplasmic polyamine-binding protein, putative	outer membrane-bounded periplasmic space; transporter activity	4.98
PP1503 PP_1503	Putative uncharacterized protein		4.86
PP3000 PP_3000	MaoC domain protein	oxidoreductase activity	4.61
PP2233 PP_2233	Hydrolase, isochorismatase family	hydrolase activity	4.36
PP3193 PP_3193	Putative uncharacterized protein		4.33
PP1524 PP_1524	rRNA large subunit methyltransferase A, putative	methyltransferase activity	4.12
PP1149 PP_1149	Putative uncharacterized protein		3.89

Table 4: Most relevant gene expression found down regulated under salt shock in P. putida (RPF 9)

ID	Name	Gene Ontology	salt shock
			Fold change
PP3349 PP_3349	Major facilitator family transporter	integral to membrane; transmembrane transport; transporter activity	-7.43
trpG PP0420 PP_0420	Anthranilate synthase, component II	anthranilate synthase activity; biosynthetic process; glutamine metabolic process	-6.83
PP1202 PP_1202	Membrane protein, putative		-6.44
PP2702 PP_2702	Porin, putative		-5.99
PP3875 PP_3875	Putative uncharacterized protein		-5.78
PP1410 PP_1410	Putative uncharacterized protein		-5.02
PP4080 PP_4080	Putative uncharacterized protein		-5.01
ltg PP3422 PP_3422	Lytic transglycosylase	lytic transglycosylase activity; membrane; peptidoglycan metabolic process	-4.95

PP3541 PP_3541	Transporter, MgtC family	membrane	-4.83
clpB PP_0625	Chaperone protein ClpB	ATP binding; cytoplasm; nucleoside-triphosphatase activity; protein processing; regulation of transcription, DNA-dependent; response to heat; transcription factor binding	-4.81
PP5147 PP_5147	Hydrolase, haloacid dehalogenase-like family	phosphatase activity	-4.53
PP2699 PP_2699	Putative uncharacterized protein		-4.48
trmD PP_1464	tRNA (guanine-N(1)-)-methyltransferase (EC 2.1.1.31) (M1G-methyltransferase) (tRNA [GM37] methyltransferase)	RNA binding; cytoplasm; tRNA (guanine-N1-)-methyltransferase activity	-4.34
fliN PP4357 PP_4357	Flagellar motor switch protein FliN	bacterial-type flagellum basal body; chemotaxis; ciliary or flagellar motility; membrane; motor activity	-4.32

It was interesting to note that, though both the stress was implicated separately, some of the genes were expressed both in case of heat shock and salt shock. AlgK and trkH were absent under normal growth conditions but they were up regulated under both stress. DnaK, a heat stress related protein were expressed in higher folds (5.19) under heat shock followed by IbpA which had 4.06 fold increase in heat shock conditions and 2.09 folds under saline shock conditions. ClpB gene was up regulated under heat stress whereas its expression was down regulated under salt stress. Five genes which were selected from transcriptome profile of heat stressed *P. putida* showed its expression comparatively in lesser folds in transcriptome profile of saline stressed *P. putida* (Table. 5). Among the five genes studied, toluene tolerance protein was expressed in higher fold under saline stress conditions. Eight genes identified from the transcriptome profile of *P. putida* were also observed under heat shock profile. ABC transporter protein was expressed highest under salt shock with 5.58 fold increases in its expression followed by toluene tolerance ABC efflux transporter showing was down regulated under heat shock stress. Six proteins which were expressed only under saline stress included trehalose synthase, Ben F like porin protein, amino acid permease protein, universal protein and Na+/Pi cotransporter family protein. The salt shock induced the expression of trehalose synthase, amino acid permease and Na+/Pi cotransporter family protein which were not initially expressed under normal growth conditions.

Table 5: Expression patterns of selected genes exposed to heat shock and salt shock analysed under Microarray

Stress	Gene	Fold change heat shock	Fold change salt shock
Expressed	dnaK	5.19	1.17
under	RNA polymerase sigma	3.09	1.02
both	clpB	1.74	-4.81
stress	toluene tolerance protein	3.32	1.11
	IbpA	4.06	2.09
	Toluene tolerance ABC efflux transporter	3.32	5.58
	glycine betaine	1.04	1.92
	N-carbomyl	1.67	1.83
	AlgK	1.74	1.91
	trkH	1.02	1.90
	polysaccharide	-1.04	1.82
	betaine alehyde	2.12	1.04
	Cysteine desulfurase	2.64	1.02
	ABC transporter, ATP-binding protein	-1.79	5.58
Expressed	DjlA	1.25	-
under	dnaJ	4.27	-
heat shock	GroEL	3.85	-
	GroES	3.32	-
	33kDa chaperonin	1.61	-
	GrpE	-2.13	-
Expressed	trehalose synthase	-	1.05
under	BenF protein	-	1.84

salt shock	amoniacid permease	-	1.91
	Universal protein	-	-1.74
	Na+/Pi cotransporter family protein	-	1.52

c) *Analysis of stress related genes coding for proteins using RT PCR*

Among the 5338 genes expressed 25 genes from both the stress conditions were selected for further studies. We found that out of these 25 genes, 14 were found to be expressed under both stress conditions, six genes under heat shock and five genes were individually expressed (Fig.2). Primers were custom designed for amplification of these selected genes expressed under heat shock and salt shock (Table. 6 and Table.7) Gyrase gene (gyrA) was selected as housekeeping gene. The data of the RT PCR obtained from were analysed. GyrA gene was used for endogenous control and samples from untreated conditions were considered as calibrators.

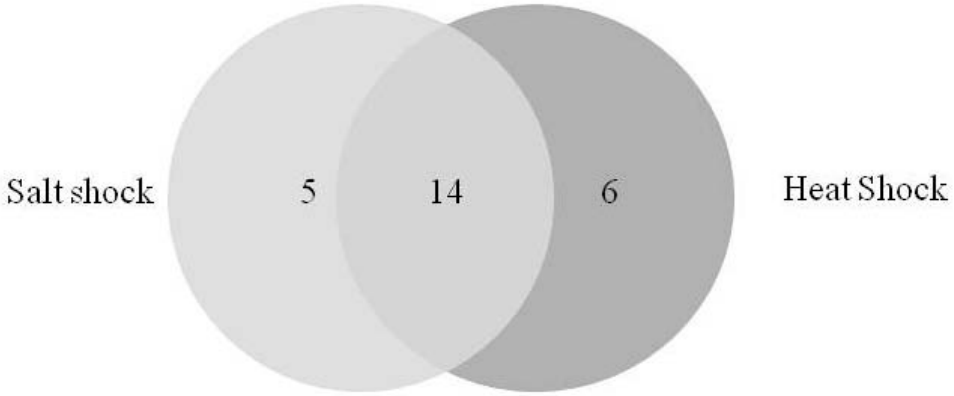

Fig. 2: Venn diagram for the selected genes and its expression pattern

Table 6: Details of primers designed for genes expressed under heat shock conditions by P.putida (NBAII RPF 9)

No.	Protein	Accession No.	Primers	
			Sense	Antisense
1	Chaperone protein DnaK (HSP70	Q88DU2	TCGACGGTGAGCACCAGTT	GCAGGGCCAGAGGATCGT
2	Chaperone protein DnaJ	Q88QT1	TTGCAAGGCTGGGAAGACATG	AGGTCCAGGCGCACCATCT
3	60 kDa chaperonin (GroEL protein)	Q88DU3	GGCGCTGACACCGAGATC	GGCCAGACGCTCTTGCA
4	10 kDa chaperonin (GroES protein	Q88N56	GCCGCTGAAAAACCAAACC	GAAAACCACTTTGTCACCCACTT
5	33 kDa chaperonin (Heat shock protein	Q88R81	GCGATTTGCCTGATACCGATT	AACACCGCGGCGTAGCT
6	Protein grpE (HSP-70 cofactor)	Q88DU1	GTGTCGAGCTGACCCTGAAGA	TGGTGGTGCTCAGGGTTGA
7	Heat-shock protein IbpA	Q88LF1	GCTGAAGTGACCTACCTGCA	CAGGCACGATGCGCAGCAGGTCGA
8	RNA polymerase sigma factor	Q88QU7	CGGAAGAGCTGGGCGAGCG	TCGATCGGGGACTGCATGGTC
9	DnaJ-like protein DjlA	Q88QT1	CACGCGGCGGAAGGTACCT	GCTCGTACTCCAGGGACAT
10	Chaperone protein ClpB	Q88Q71	CTTCCGCAACACCGTGATCG	ATGAATTCCGGACGGAAGTG
11	Toluene-tolerance protein	Q88P90	GTGCTGATCGATGGCTCG	TCGCACGCCTTGCCAGT

Table 7: Details of primers designed for genes expressed under salt shock conditions by P.putida (NBAII RPF 9)

No.	Protein	Accession No.	Primers	
			Sense	Antisense
1	Housekeeping gene, gyr A	X54631	AGACCTACGTTCACGGCGTA	GAACGACAACTCGCGAATAC
2	Glycine betaine/L-proline ABC transporter, permease protein	Q88R39	GTGTCACTCAATGCATCAT	GGGAGATATCGGCGGTGTT
3	Trehalose synthase, putative	Q88FN0	GTACTTCTGGCACCGGTTCT	CTCGATCAGGTAAGGGATC
4	N-carbamoyl-beta-alanine amidohydrolase, putative	Q88FQ3	GACCTGGGCGTGGAAACC	GGTGTCTTCCAGGGTGAACTTG
5	BenF-like porin	Q88JX6	AAGGCAAAGCCGAGGAATG	TAAAGGCCCAGCACGTCAAC
6	Amino acid permease	Q88DR7	GTTCATCACTGTGATGTTC	AGATCGACAGGATGTAGAAG
7	Alginate biosynthesis protein AlgK	Q88NC7	TGACAGCGTGGCCCGGGTGCT	GAAGTCGTATACCAGCTG
8	Potassium uptake protein, TrkH family	Q88FX4	TCGTCTGGCTCACCGTCG	CAGAGTGTGTAATCACCCA
9	Polysaccharide biosynthesis protein	Q88LX2	AGTTGATTCTGCAAGCACTA	ATCACCGAGCCAGAGGAG
10	Na+/Pi cotransporter family protein	Q88RI4	TCATCAGTACCAGCATGCAGAA	CGCAGCGTGTTGTAGAGCAG
11	Betaine aldehyde dehydrogenase, putative	Q88MT7	GCATCCGCACCTTCGGCTTCT	CTGGCTACGGCTTTACCCAGGCG
12	Universal stress protein family	Q88KV2	CCTGCATGCCAGCATCATCG	GATCGTATCGCTCAGCTGGA
13	Toluene tolerance ABC efflux transporter, ATP-binding protein	Q88P94	CGTGCATACTCAGCTGTCG	CCAGCGCCACACGGCGCTTCAT
14	Cysteine desulfurase	Q88K56	CAGATAGCGGGCATGGGCAGT	TGCTTAGCGCAGCCGTTCAGG
15	ABC transporter, ATP-binding protein	Q88HZ1	GTGTCACTCAATGCATCAT	GGGAGATATCGGCGGTGTT

The Relative quantification of target gene was calculated based on its expression in non stressed conditions to stressed conditions. The results summary of the quantification of target genes with their Relative Quantification (RQ) value for the genes expressed under heat shock is represented in Table 8. The highest fold change of 13.67 was observed in IbpA gene followed by RNA polymerase sigma factor which was expressed under heat shock conditions. Major heat stress related proteins like dna and dnaJ were slightly up regulated, meanwhile, GroES, GroeEL and Hsp33 were upregulated two fold more under heat shock stress by *P. putida* (NBAII RPF 9). Among 11 heat shock related proteins analysed, djlA was slightly down regulated.

Table 8: Quantification of heat shock related genes tested using RT PCR

Sample	CT (Mean)	CT (Std Dev)	Δ CT (Mean)	Δ CT (Std Err)	ΔΔ CT	RQ	RQ (Min)	RQ (Max)
gyrA‡								
Sample 1*	22.997	0.4286						
Sample 2	24.7793	0.1983						
IbpA								
Sample 1*	24.9171	0.011	1.9201	0.2476	0	1	0.5792	1.7266

Sample 2	22.926				-3.7734	13.6741		
Sig								
Sample 1*	18.7844	0.197	-4.2125	0.284	0	1	0.5345	1.871
Sample 2	18.6855	0.0434	-6.0938	0.1435	-1.8813	3.6839	2.4012	5.6519
clpB								
Sample 1*	28.8711	0.0598	5.8742	0.2511	0	1	0.5748	1.7398
Sample 2	28.9032	0.0698	4.124	0.1486	-1.7502	3.3641	2.1596	5.2404
Ttp								
Sample 1*	22.8046	0.1032	-0.1923	0.258	0	1	0.566	1.7688
Sample 2	22.9226	0.5663	-1.8566	0.4243	-1.6643	3.1696	0.8942	11.2348
djlA								
Sample 1*	20.8755	0.15332	-4.5524	0.265	0	1	0.6005	1.6653
Sample 2	20.8544	0.1357	-4.5488	0.3088	0.0036	0.9975	0.5506	1.8073
dnaK	21.8402	0.0734	-2.7377	0.1765	0	1	0.7119	1.4046
Sample 1*	21.7747	0.1507	-3.048	0.204	-0.3103	1.24	0.8374	1.8361
Sample 2								
dnaJ								
Sample 1*	20.558	0.2356	-4.02	0.2185	0	1	0.6568	1.5226
Sample 2	20.4092	0.4243	-4.4123	0.0367	-0.3935	1.3136	0.728	2.3702
GroEl								
Sample 1*	26.9449	0.0974	2.367	0.1804	0	1	0.7067	1.415
Sample 2	25.894	0.395	1.0727	0.2933	-1.2943	2.4525	1.3946	4.313
GroES								
Sample 1*	28.5046	0.2374	3.9266	0.2195	0	1	0.6555	1.5256
Sample 2	27.2841	0.3214	2.4614	0.2617	-1.4652	2.7611	1.6687	4.5684
Hsp33								
Sample 1*	28.6233	0.3699	4.0453	0.2739	0	1	0.5904	1.6939
Sample 2	27.4897	0.8704	2.667	0.5353	-1.3784	2.5997	0.9279	7.2838
GrpE								
Sample 1	19.885	0.074	-4.693	0.1766	0	1	0.7118	1.4049
Sample 2	19.5331	0.0959	-5.2859	0.1926	-0.5966	1.5121	1.0437	2.1907

(∓) denotes endogenous control
(*) denotes calibrator

The relative quantification of stress tolerant genes produced by *P. putida* (NBAII RPF 9) under salt shock showed≤ 5 folds increase in expression of toluene tolerance proteins followed by alginate biosynthesis gene which recorded 4.01 fold increase in its expression under salt shock compared to its expression under non stressed condition (Fig. 3). More than 50% of the saline stress related protein quantification recorded two fold of their expression under salt shock stress than in non stressed conditions. This expression of these genes may not be imperative under normal growth conditions but they were triggered under salt stress as a protective mechanism of *P. putida* (NBAII-RPF 9) to surmount the detrimental effects on its survival. Glycine betaine/L-proline ABC transporter was slightly down regulated under salt shock.

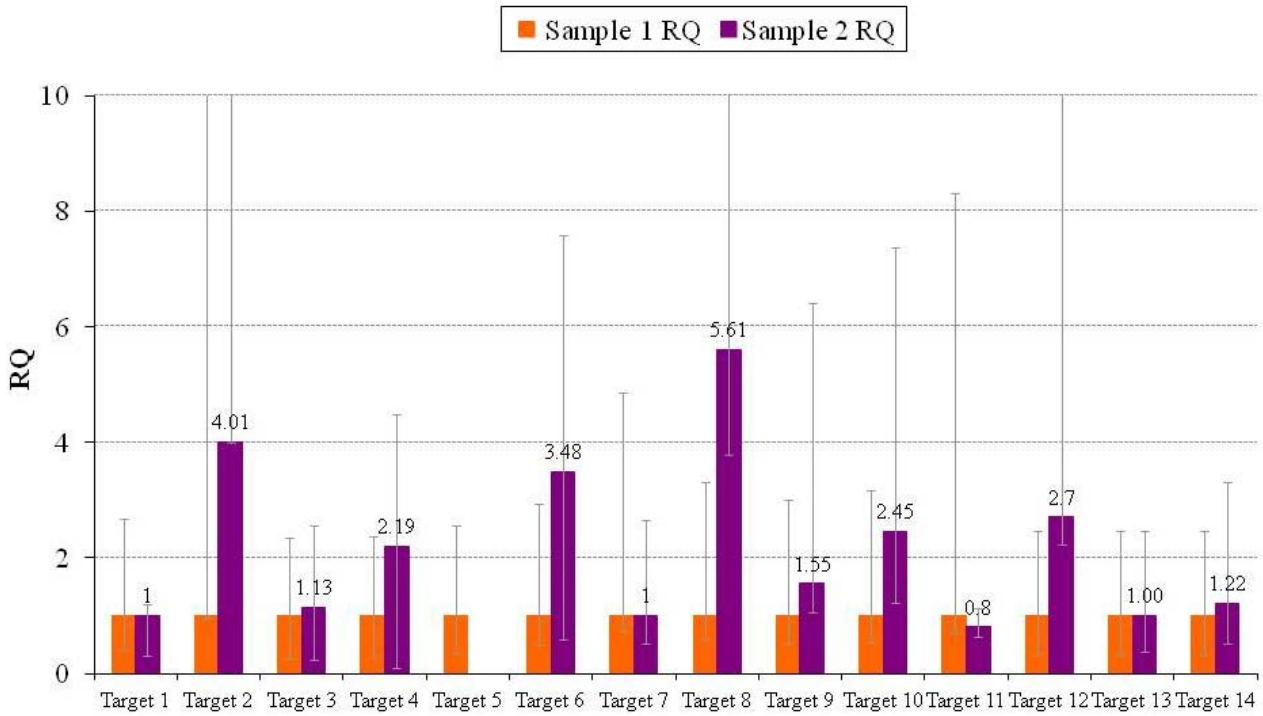

Fig. 3: Graphical representation of RQ values of genes expressed under saline stress by *Pseudomonas putida* (NBAII RPF 9). Target1: Amino acid Permease, Target 2: AlgK, Target 3:TrkH, Target 4: Polysaccharide biosynthesis protein, Target 5: Na+/Pi cotransporter family protein, Target 6: Betaine aldehyde dehydrogenase, putative, Target 7: Universal stress protein, Target 8: Toluene tolerance, Target 9: cysteine dehydrogenase, Target 10: ABC transporter, Target 11: Glycine betaine/L-proline ABC transporter, Target 12: Trehalose synthase, Target 13: N-carbamoyl-beta-alanine amidohydrolase, Target 14: BenF-like porin.

IV. Discussion

Microarray is high throughout technology which has enabled specific and sensitive methods for environmental studies and its response to environmental cues (Zhoa, 2003). Based on our literature survey, we were aware that the number of genes expressed during sudden stress was more compared to steady state stress induced. Hence, we preferred to induce a sudden heat shock and saline shock which would probably increase our chances of finding gene expression far more than in steady state conditions. This was even proved in case of our proteomic studies where we found more expression of proteins under abiotic shock rather than steady state stress. Both NaCl and PEG 6000 result in sequestration of water and changing water potential out of the cell. Hence we used NaCl as a medium to induce osmotic stress. The fold induction of stress related proteins were more or less similar (Peterson, 2009). In our earlier studies we have isolated and screened different strains of *Pseudomonas* having varied stress responses. *P. Putida* were able to survive under high salinity and high temperature stress whereas *P.* fluorescens survived better in water limiting stress (Rangeshwaran *et al*., 2013; Ashwitha *et al*., 2018). Choudhury *et al*. (2019) observed that *P. fluorescens* revealed resistant traits against oxidative stress compared to *P.* aeruginosa which were sensitive. Salt stress also creates a major impact on soil microbes however, some of the bacterial species mitigate these stress by accumulation of low molecular weight hydrophilic molecules (Kempf *et al*., 1998). In response to hyperosmotic stress, bacteria adjust their cell turgor by controlling fluxes of ions across cellular membranes. Osmoprotection by compatible solutes and the general stress response are linked, because the structural

genes for the proline uptake system OpuE (Spiegelhalter and Bremer, 1998) and the glycine betaine transporter OpuD (Kappes *et al*., 1996) are partially dependent on SigB for their expression.

P. putida KT 2440 is the only *Pseudomonas* strain whose whole genome is sequenced and thus it serves as a standard reference for most of the transcriptomic and proteomic studies. In our studies, we customised the microarray slides by hybridising *P. putida* KT2440 genes. Ballerstedt *et al*. (2007) have described the functional genes of *P. putida*. Hence, use of this reference strain has increased the chance of highly sensitive and reproductive microarray analysis with our isolate of interest *P. putida* (NBAII RPF 9). Salt stress induces opulence of events like changes in cell membrane physiology, exopolysaccharides structural content, membrane composition and homeostasis. *Bacillus* sp. exhibited up regulation of cell envelope proteins, molecular chaperones, compatible solutes to impart tolerance to high salt/osmolarity stress (Yin *et al*., 2015). Wang *et al*. (2011) were successful in an attempt to detect genes in degradation of 3CB by isolation of RNA from sterile soil treated with *P. putida* KT2440 to find expression of gene in transport and stress response. This provides an insight that microarray analysis of *P. putida* response can be evaluated even when evaluated directly from environmental samples.

The Microarray data helped us in selecting 25 stress related genes which were quantified using RT PCR. Gyrase gene was selected as housekeeping genes. McMillan *et al*. (2014) have quantified the genes responsible for *Azospirillum* cells to turn into cyst like form when stress was induced using qRT-PCR using gyrase as reference gene. Their studies revealed that stably expressed reference genes viz., gyrA, glyA and recA were suitable for normalization of qRT PCR data in *A. brasilense* under normal and stress conditions. GryA was identified as stable reference genes in *Xanthomonas citri* (McMillam and Pereg, 2014). In our study we studied Microarray analysis by induction of stress under laboratory conditions.

The quantification was determined with input copy number by relating PCR signal to a standard curve. The RT PCR results analysis with $2^{-\Delta\Delta CT}$ for gene expression in a treatment group to that of another sample such as an untreated control (Livak and Schmittgen, 2001; Pabinger et al., 2014). Single peak in the melting curve shows efficient reaction and ruled out any non specific product. We found expressions of heat shock related proteins under saline shock (1 M NaCl) conditions. This could indicate that *P. putida* (NBAII RPF9) might provide cross protection to multiple stresses. We found a repressed activity of universal stress protein under saline shock. Universal stress proteins were observed to be expressed in *P. putida* KT 2440 to deal with survival of the bacteria under excess 3CB during growth. However, their reduction in fold change is unexplained but it is seen to be present even under stress (Wang *et al*., 2011). Genes for alginate biosynthesis are majorly reported in *P.* aeruginosa causing cystic fibrosis. Most of the transcriptome studies have been concentrated on human pathogenic *Pseudomonas* sp. as these have been a challenge due to its complex biology (Balasubramanian and Mathee, 2009). This isolate survives environmental cues by forming biofilm. AlgK was up regulated under sudden saline shock subjected to *P. putida* (NBAII RPF 9). AlgK is an outer membrane lipoprotein which is involved in localization of algE porin to outer membrane. These cell envelopes act as primary barriers against lethal effects of stresses to bacterial cells (Keiski *et al*., 2010; Maleki *et al*., 2015; Wood and Ohman, 2015).

In our study, we found 4.01 times increase in algK over control conditions. Aldehyde dehydrogenase work on mitigating external stresses like oxidative, electrophilic stresses in bacterial cells (Singh *et al*., 2012), betaine aldehyde dehydrogenase regulated production of compatible solutes viz., glycine betaine which provides tolerance to bacterial strain under osmotic stress (Boch *et al*., 1997). The BADH activity has been reported to impart tolerance to salt stress even in *P.* aeruginosa species (Velasco-García *et al*., 2006). ABC transport proteins are involved in transfer of substrates across membranes. These proteins work closely in stress conditions and regulate movement of cytotoxic compounds across cells (Nagar *et al*., 2016). We found up regulation of ABC transport proteins , Na+/Pi cotransporter family protein and Toluene tolerance ABC efflux transporter, ATP-binding protein in case of salt shock. The osmotic imbalance could affect the homeostasis of the cell. The transport proteins save the cells from osmotic breakdown. The expression of these transport proteins in *P. putida* (NBAII RPF 9) was evident for its survival under salt stress. Gulez *et al*. (2012) carried out a similar transcriptome studies to unveil the stress response of *P. putida* KT2440 which, has been used in our study to as a standard for generating micro array also exhibited expression of proteins like alginate biosynthesis at early stage of stress which can be related to osmotic shock treatment with 1M NaCl in our experiments. Apart from this protein, heat stress related proteins were also seen to down regulate under salt stress. The universal stress protein was down regulated even in *P. putida* KT2440 subjected to water stress using PEG 8000 (Gulez *et al*., 2012). Trehalose is one of the compatible solutes which maintain the imbalance of cells under salt stress majorly by providing carbon and energy source to bacterial cells under high osmolarity conditions. The release of this protein is a major mechanism in halotolerant bacterial strains. Though trehalose is observed to accumulate in osmolarity stress, it is also reported to mitigate heat stress (Reina-Bueno *et al*., 2012).

Osmoprotectants reduce the lethal effects by increasing stability of macromolecules under low water activity. Most of the studies relate that expression of stress related proteins were in higher fold under shock and slowly reduced when the stress conditions were provided in steady state. Osmolarity stress induced expression of thermo tolerance genes whereas no genes were identified in heat stress related proteins which could mitigate the osmotolerance (Gunashekara *et al*., 2008).We found the expression of heat shock responsive molecular chaperones as common under tolerance or shock. This indicates that these play a major role under shock or tolerance. All other proteins identified were different but are also involved in stress response. Most of the up regulated heat shock proteins play a pivotal role in maintaining physiological and metabolic functions. Heat stress responsive molecular chaperones like cytosolic-type hsp90, chaperone protein DnaK and heat shock 70 kDa proteinHsp90 is a highly conserved molecular chaperone involved in stabilizing and refolding of denatured proteins and is generally referred as HTPG in prokaryotic system (Challis *et al*., 2000; Rangeshwaran *et al*., 2013). The heat shock stress proteins showed high fold expression at initial levels which gradually decreased over a period and the up shift of temperature was followed by a period of acclimatization to the stress induced. Heat shock proteins like DnaK, DnaJ, GroEL, GroES and GrpE were observed to up regulated in heat shock response in *Acidithiobacillus ferrooxidans*. All these HSPs were also seen to be up regulated even in *P. putida* (NBAII RPF 9) (Xiao *et al*., 2009; Chan et al., 2016). GrpE is known to express mainly during low and high temperatures however, they have even been induced under stress induced by chemicals or others making it an essential gene for survival of bacteria under stress conditions. Zhang and Griffiths (2003) demonstrated that grip played a major role in starvation induced thermotolerance at lower temperatures in E.Coli O157:H7. The expression of grpE genes coupled with another stress related gene UspA was measured through flourescent tagging in *E.Coli*. Maximum expression of protein was observed when *P. fluorescens* were exposed to 37 °C which is above optimum temperature for the bacterium. Similar results are previously reported in our earlier research. *P. putida* and *P. fluorescens* have increased expression of heat stress related proteins, metabolism regulating proteins and outer membrane proteins (Arana *et al*., 2010; Rangeshwaran et al., 2013).

Thermal shock induced in biofilm forming *P. aeruginosa* showed decrease in population to almost nil after exposure to high temperatures for more than 1 minute (Ricker *et al*., 2018). Contradictorily in our results, *P. Putida* (NBAII RPF9) showed decreased population however, they were culturable even after prolonged exposure to heat (Ashwitha *et al*., 2013; Rangeshwaran *et al*., 2013). In our earlier studies we have demonstrated the selection of abiotic stress tolerant *Pseudomonas*. Their proteomic studies have revealed an expression cocktail of stress related genes which could possibly be a major reason for *P. putida* to be able to survive under high temperature and high saline stress. In current research we have attempted to under the mechanism of stress tolerance of *P. putida* strain when subjected to heat shock and saline shock by analysing its expression pattern using transcriptome studies. The majorly expressed genes were quantified and analysed. The study showed the expression of a few genes already reported and some of them which is reported for the first time. The transcriptome analysis using microarray and further validation with RT PCR technique was useful in investigating the gene expression profiling of *P. putida* (NBAII RPF 9) and helped us in unravelling the mechanism by which the isolate survived under abiotic stress.

V. Conclusion

Pseudomonas is one of the ubiquitous microorganisms with immense demand in agricultural applications. Different species of *Pseudomonas* have been explored for more than three decades for its use in plant health improvement and disease management. Soil dwelling *Pseudomonas* are importantly isolated and used as bio agent for agricultural applications. Soil is a complex ecosystem and constantly exposed to innumerable stress due to natural and human activities. This impacts the *Pseudomonas* sp. which are the one the major constituents of the soil. The ability for the micro-organisms to cope with these stresses are its survival agenda. Though biocontrol research has focused on selection and application of soil dwelling *Pseudomonas* with excellent plant growth promoting properties, their functioning and effects have never been satisfactory during the application in different field ecosystems. During these years, the research community have come across challenges to decode the lack of performance of most of the best strains when applied to different ecosystems. The advanced biocontrol technologies have created a parallel way of analysis and selection of potential strain which can perform well in normal as well as stressed environmental conditions. The development of new age techniques like microarray and quantitative RT PCR have enabled scientists to dig deep and understand how a bacterium responds when it is exposed to different environmental cues thereby selecting a versatile strain.

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Intestinal Helminths Infections and Predisposing Factors among Pupils from Selected Primary Schools in Kamuganguzi Subcounty, Kabale District, South Western Uganda

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Methods: It was a descriptive cross sectional study design where both qualitative and quantitative approaches were employed. Pupils (100) were randomly selected from each of the eight selected primary schools. A structured questionnaire was used to capture information associated to risk factors and 798 stool specimens were collected and examined for intestinal parasites by microscopy directs a line wet-mounts and formol ether concentration technique in the laboratory. Data was analyzed using statistical software SPSS version 20.

Keywords: *intestinal helminths, prevalence, south western uganda, pupils.*

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Results: Out of 798 stool specimens collected and examined, 22.3% (178/798) tested positive for intestinal helminths with *Trichuristrichiura* and *Ascarislumbricoides* responsible for the cases. *Ascarislumbricoides* was the most prevalent with 96.7%, *Trichuristrichiura* with 3.3 % whereas 1.7% of the pupils had mixed infection of both *Ascarislumbricoides and Trichuristrichiura*. Anal cleaning practices (p-value 0.018) and water sources (p-value 0.002) were the main predisposing factors for the spread and maintenance of helminths infections in school going children in the study area.

Conclusion: Intestinal Helminths infections remains a health challenge among school going children in Kamuganguzi sub county; where *Ascaris lumbricoides and Trichuris trichiura* are responsible for infections. Poor anal cleaning practices and unreliable sources of water are the main predisposing factors for transmission and maintenance of Helminths infections in the study area.

Recommendations: There is need to increase access to safe and reliablewater sources in schools and to integrate anti-Helminths drugs admission with water treatment and community sensitization programs if parasitic infections are to be contained in this area.

Keywords: intestinal helminths, prevalence, south western uganda, pupils.

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I. Introduction

Intestinal helminths are intestinal parasites that inhabit the human gastrointestinal tracts and are one of the most prevalent forms of parasitic disease causing organisms attributed to physical and intellectual growth retardation (Abera & Nibret, 2014).Intestinal helminthiasis is caused by different species of worms especially in the group of round worms which include *Ascaris lumbricoides, Trichuris trichiura,* Hookworms *(Necator americanus* and*Ancylostoma duodenale), Strongyloides stercolaris, Taenia*species and schistosomes (Cheesbrough, 2005).

In sub Saharan Africa, intestinal helminth infections are common and of major health concerns because factors that predispose man to the infections are bound in the sub-region (Ijagbone & Olagunju, 2006).). Intestinal parasitic helminth infections are a major health challenge in primary school going children especially in developing countries (Mirisho, Neizer & Sarfo, 2017). Intestinal parasites cause high morbidity and mortality throughout Uganda (Hemant Kumar, 2014). This was associated with poor sanitation, lack of access to clean water, inadequate health facilities, and poverty (Adu-Gyasi et al., 2018).These infections cause iron deficiency anemia, growth retardation in children and other physical and mental health problems (WHO, 2012). About 610 million school age children worldwide are at risk of infection with common intestinal (Teshale *et al.*, 2018); most prevalent in the tropical and the subtropical areas where adequate clean water and sanitation facilities are lacking (Sun, 2015).

In Uganda, the prevalence of intestinal helminths infections among children is high estimated to be 82.1% due to *Hookworm,*18.9% *Ascaris lumbricoides*, 7.0% *Trichuris trichiura*, 1% *Enterobius vermicularis* and 0.5% for *H. nana* making it one of the leading countries with these infections in the world despite the government's efforts in the control(MoH Report, 2010). The study in selected districts of Central, Eastern, Northern, Karamoja, Westnile and Western regions where the most common STH were *Hookworms* (7.7%), *Trichuris trichiura*(1.3%) and *Ascaris lumbricoides*(0.5%).

Bundibugyo District had the highest prevalence infection with the prevalence of 24.9%, 4.1%, 4.1% and 28.2% for *Hookworm, Ascaris lumbricoides,Trichuris trichiura* and any infections of STH respectively (Adriko*et al*., 2018). In Hoima district, rural western Uganda, the prevalence is 26.5% (149/562); the prevalence of respective parasites included Hookworm 18.5% (104/562), 9.8% (55/562) for *A. lumbricoides* and 0.5% (03/562) for *T. trichiura (*Ojja *et al.,* 2018); While in Wakiso District-Central Uganda was 10.9% for *hookworm*, 3.0% for *Trichuria trichiura*, 1.9% for *Schistosoma mansoni* and 0.2% for *Ascaris lumbricoides* respectively (Lwanga,Kirunda & Orach, 2012).

A number of pupils in this study area have been noticed complaining of abdominal pains with the majority presenting with diarrhea (Kamuganguzi HC IV registers for the year 2018-2019).They do not routinely wash their hands after the latrine use and or before eating due to insufficient hand washing facilities and often defecate in the open due to inadequate latrine coverage; many pupils present with signs and symptoms of intestinal helminthiasis and cases have been reported among school going children in Kamuganguzi community. However, there is no clear documentation about the magnitude and predisposing factors of intestinal helminths infections in this area.

This study therefore sought to determine the prevalence of intestinal helminths infections and to establish the predisposing factors among pupils in selected schools of Kamuganguzi Sub County in Kabale, Western Uganda to fill this information gap. These findingstherefore enhance the level of awareness about the burden of intestinal helminthias is and predisposing factors key in guiding planning, broadening of control and preventive measures for intestinal helminths infections.

II. Materials and Methodology

a) Study area and design

The study was a laboratory and school based study using a descriptive cross sectional study design where both qualitative and quantitative approaches were employed.The study wascarried out inprimary schools of Kamuganguzi sub county, Kabale District, south western Uganda.

It is located on Kabale-Katuna highway, about 18 km from Kabale town and 2.5 km to Katuna border. Kabale District is located is in south western Uganda. it is a highland District at the coordinates $01^0$15`S $30^0$00`E with the altitude ranging between 1219metres (3999ft) and 2347metres (7700ft) above sea level. This altitude makes the area to be colder than rest of the country with a temperature average of 18^0C (64^0F) during the day and fall to about 10^0C (50^0F) at night. The relative humidity of the area is between 90% and 100% in the morning and decreases to between 42% and 75% in the afternoon all the year around.This sub county has eleven primary schools which include; Buhumba, Katenga, Kicumbi, Buranga, Kasheregyenyi, Kasaasa, Bunagana, Kikore, Kyasano, Butuza and Rutare primary school with a population size of about ten thousand pupils.

b) Sample size and study population

The sample size was 798 pupils estimated using Kish formula (Kish L, 1965) taking precision assumed to be +/- 0.05 at 95% level of confidence and taking 26.5% as prevalence of intestinal helminth infections in Hoima (Ojja *et al.,* 2018).This study included pupils from the selected primary schools of KamuganguziSub County, Kabale district.

The sub county has eleven primary schools which include; Buhumba, Katenga, Kicumbi, Buranga, Kasheregyenyi, Kasaasa, Bunagana, Kikore, Kyasano, Butuza and Rutare primary school. Eight primary schools were randomly selected from the eleven schools.The study considered pupils whose teachers/administration accented and those who met the selection criteria among the selected primary schools.

c) Sampling techniques

Simple random sampling technique was used to select eight schools from the eleven schools that are in Kamuganguzi Sub County. Papers were labeled 1-8 and other three papers were left blank. These papers were folded and put in a basin. Researchers then selected 11 teachers, at least one from each of the primary schools in Kamuganguzi Sub County and subjected them to picking one paper randomly from the pool of papers placed in a basin. Teachers who picked papers with numbers had their schools allowed to participate in the study.

Pupils were randomly sampled from every class including both boys and girls across the selected primary schools using class register. A maximum of one hundred pupils were selected from each school to participate in the study.On each day 100 stool specimens were collected from a single school and the samples analysed on the same day.

A pre tested, standardised interview guide was used to obtain the qualitative information from teachers and guardians about factors predisposing children to intestinal helminthic infections and also to collect demographic information concerning the study participants after obtaining the informed consent. For easy understanding of the questions asked to them, the questions were translated into Runyankole which is a native local language.

d) Sample collection and laboratory procedures

Researchers briefed and gave instructions of specimen collection to all study participants before specimen collection. All samples were collected aseptically in sterile, leak proof and labelled

containers.Samples were examined from Kabale Regional Referral Hospital Laboratory; stool samples were analysed macroscopicallyfor consistency, colour and for the presence of adult parasites and microscopically in direct wet techniques and formal ether concentration technique. Specimens were preserved in 10% formal saline.

e) Ethical considerations

The study was approved by Research Ethics Committee and Faculty of Medicine Research Ethics committee (Ref: MUST/MLS-031) at Mbarara University Science and Technology. Permission was sought from the district health officer Kabale district and from head teachers of selected schools. Informed consent was sought from all study participants, and their information was treated with confidentiality.

f) Quality control

Samples were transported to Kabale Referral Hospital for analysis and examination at ambient temperatures as soon as they were collected .Visual aids such as coloured plates and charts were used toconfirm the identity of the parasites or unusual structures.10% of examined specimen were proof read by a senior medical laboratory scientist for quality control

g) Data analysis and presentation

Data collected was cleaned and checked for completeness, errors, and consistence, entered in excel and transferred into SPSS version 20 for for descriptive data analysis and the outputs presented in frequency tables and graphs. Bivariate analysis was run using chi-square to establish factors associated with the prevalence of intestinal parasites. Factors with a p-value of 0.05 were considered statistically significant. The prevalence of intestinal parasitic infection was calculated and presented in frequency, percentages and table form.

III. Results

a) Socio-demographic characteristics of study participants

Seven hundred ninety eight (798) pupils were selected from eight primary schools in Kamuganguzi Sub County and interviewed with the aid of questionnaires to capture socio-demographic characteristics as shown in table 1.

Table 1: Socio-demographic characteristics of study participants

Variables	Frequency(n=798)	Percent (%)
Gender		
a) Male	372	46.6
b) Female	426	53.4
Age group (Years)		
a) <5	24	3
b) 6-10	456	57.1
c) 11-12	187	23.4
d) >12	131	16.4
Name of school (Primary)		
a) Kamuganguzi	100	12.5
b) Katenga	100	12.5
c) Buranga	100	12.5
d) Bunagana	100	12.5
e) Butuuza	100	12.5
f) Kisasa	100	12.5
g) Kasheregyenyi	99	12.4
h) Kicumbi	99	12.4
Total	798	100

Majority of study participants were female 53.4% (426/798); and belonged in 6 – 10 age group contributing 57.1% (456/798) of study participants.

b) Laboratory Findings

Prevalence of Intestinal helminthiasis and Helminths species isolated

From the laboratory findings, only two helminths species were isolated that is *Ascaris lumbricoides* and *Trichuris trichiura* with different proportions. Out of 798 stool specimens examined, 175/798 were positive for *Ascaris lumbricoides* and 06/798 were positive for *Trichuris trichiura* giving a prevalence of 21.9% and 0.75% respectively (Figure 1).

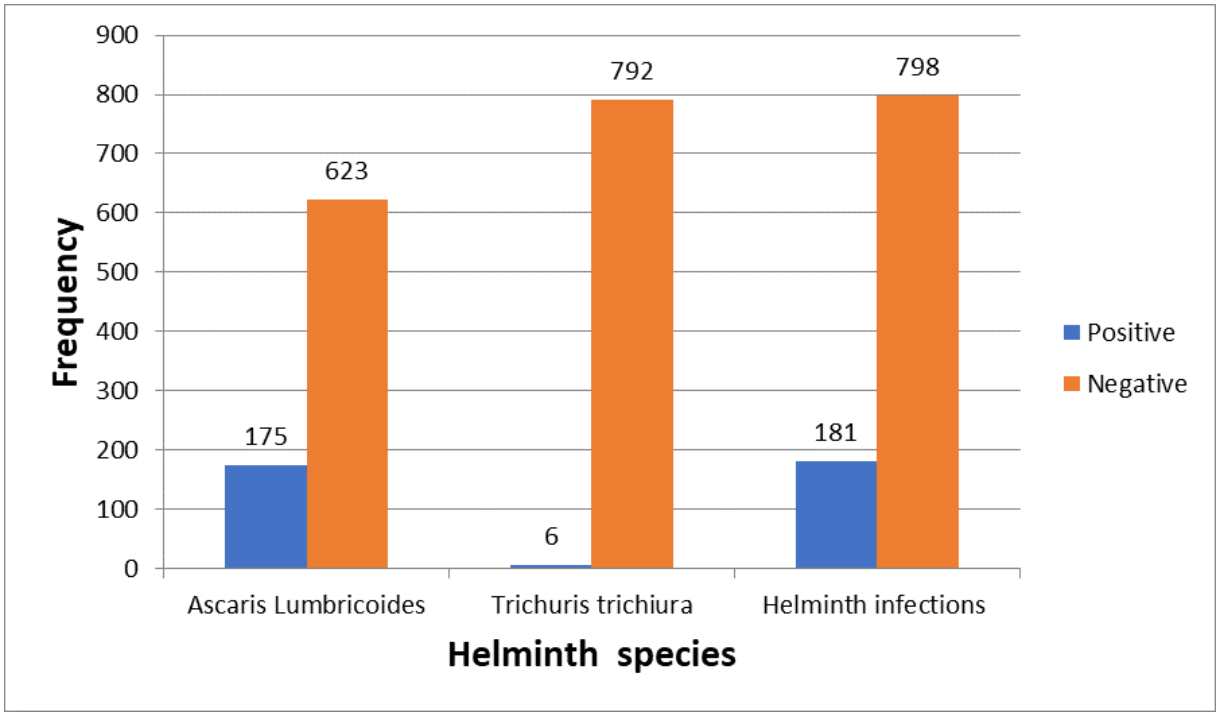

Figure 1: A bar graph showing the prevalence of intestinal helminths species isolated

Out of 798 stool specimens examined, 181/798 tested positive for intestinal Helminths infections giving an overall prevalence of 22.7% (Figure 2).

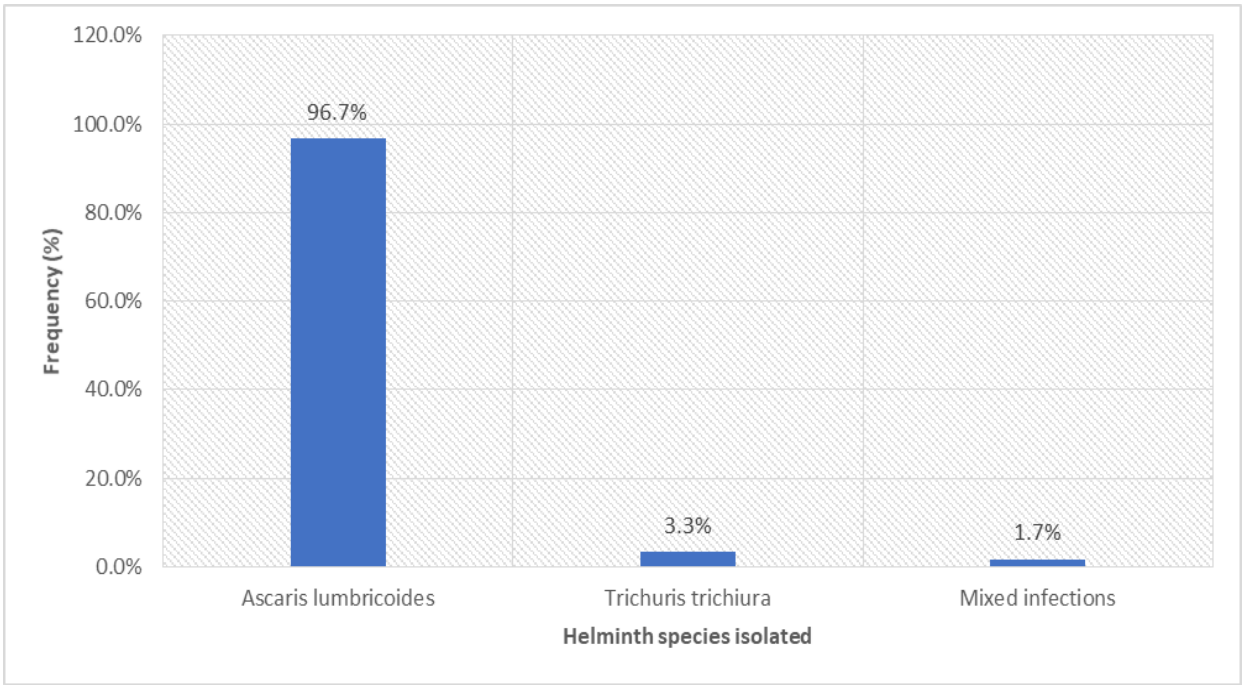

Figure 2: Shows the proportion of different intestinal Helminths responsible for intestinal Helminths infections

c) Most prevalent Intestinal Helminth species

Ascaris lumbricoides was the most prevalent helminths species responsible for 96.7% of all infections identified. However, 1.7% of mixed infections of both *Ascaris lumbricoides*and *Trichuris trichiura* were observed.

d) Predisposing factors to Intestinal Helminths infections

A questionnaire was used to capture predisposing factors and then a Chi square test carried out to determine the association helminths infections and to establish the level of significance (Table 2).

Table 2: The predisposing factors for intestinal Helminths infections among study participants

Variable	Frequency	Helminth infection		P-value
	Total (n)=798	Negative (%)	Positive (%)	
Gender				
Male	372	288(77.4)	84(22.6)	0.862
Female	426	332(77.9)	94(22.1)	
Age group (Years)				
<5	24	20(83.3)	4(16.7)	0.396
6-10	456	362(79.4)	94(20.6)	
11-12	187	138(73.8)	49(26.2)	
>12	131	100(76.3)	31(23.7)	
Defecation points				
Pit latrine	763	594(77.9)	169(22.1)	0.788
Stream	1	1(100)	0(0)	
Bush	33	24(72.7)	9(27.3)	
Bush and pit latrine	1	1(100)	0(00)	
Anal cleaning				
Plant Leaves	514	385(74.9)	129(25.1)	0.018
Donot clean	38	32(84.2)	6(15.8)	
Use papers	65	59(90.8)	6(9.2)	
Toilet papers	181	144(79.6)	37(20.4)	
Hand washing				
No	324	256(79)	68(21)	0.46
Yes	474	364(76.8)	110(23.2)	
Wash hands				
Before eating	1	1(100)	0(0)	0.814
After toilet	132	101(76.5)	31(23.5)	
Before food and after toilet	335	257(76.7)	78(23.3)	
Not applicable	330	261(79.1)	69(20.9)	
Materials used				
Water only	245	184(75.1)	61(24.9)	0.487
Soap and water	221	173(78.3)	48(21.7)	
Not applicable	332	263(79.2)	69(20.8)	
Water source				
Pond	316	228(72.2)	88(27.8)	0.002
Stream	208	168(80.8)	40(19.2)	

Tap	271	223(82.3)	48(17.7)	
Well	3	0(00)	3(100)	
Boiling water				
No	300	240(80)	60(20)	0.225
Yes	498	380(76.3)	118(23.7)	
Taking deworming tablets				
No	362	287(79.3)	75(20.7)	0.326
Yes	436	333(76.4)	103(23.6)	
When last took tablets				
1 month ago	25	21(84)	4(16)	0.301
Few days back	11	10(90)	1(19.1)	
Don't remember	161	116(72)	45(28)	
3 month ago	117	88(75.2)	29(24.8)	
6 month ago	14	97(80.2)	24(19.8)	
Not applicable	363	288(79.3)	75(20.7)	

Anal cleaning practices (P-value 0.018) and water sources (P-value0.002) were the only statistically significant predisposing factors associated to intestinal helminths infections in the study. There was no statistically significant association between age group, sex of children, hand washing, when washing hands, boiling water, taking tablets though they were reported to be contributing to the spread of Helminth infections.

IV. Discussion

Intestinal Helminths infections remains a health challenge among school going children in Kamuganguzi sub county; where *Ascaris lumbricoides and Trichuris trichiura* are responsible for infections. Poor anal cleaning practices and unreliable sources of water are the main predisposing factors for transmission and maintenance of Helminths infections in the study area

a) Prevalence of intestinal Helminths infections

Out of the total 798 pupils that were enrolled in our study, the overall prevalence of intestinal helminth infection was found to be 22.7%. This is slightly lower when compared to the studies carried out by Ojja *et al.,* (2018) in Uganda which showed that the prevalence of intestinal helminthes among the preschool-age children in Hoima District was 26.5%. The difference in prevalence could be due to the existing regular deworming programs in our study area. However, Mote, Makanga & Kisakye, (2005) in their study carried out in West Nile region Uganda to determine the prevalence of intestinal parasites among school children in Moyo district, found out that 42.6% were infested with at least one or a combination of up to three types of helminths which is higher than the 22.7% prevalence in our study area. The high prevalence in Moyo might be due to the climatic and environmental conditions of the area which could be more favorable for intestinal helminths since the temperatures are higher in Moyo than it is in Kabale and to some extent attributed to timing and seasonal differences in conducting the study.

According to other studies that were done among schoolchildren indifferent countries, the prevalence in this study is higher than that obtained by Mirisho, Neizer & Sarfo, (2017) among Children Attending Princess Marie Louise Children's Hospital in Accra, Ghana which was found to be 17.3% (39/225). Their low prevalence might be attributed to the laboratory method used since they did not use any concentration technique to increase chances of ova detection. However, Wani & Amin, (2016) in a study carried out among children of Kashmir valley in India found out that the prevalence of intestinal helminths was 75.28% which is very high as compared to our finding. The higher prevalence in their study is strongly attributed to the indiscriminate defecation of their study participants.

b) The most prevalent intestinal Helminths

Our study indicated that *Ascaris lumbricoides*was the most prevalent with 96.7% followed by *Trichuris trichiura* with 3.3 %. However, 1.7% of the pupils had mixed infection of both *Ascaris lumbricoides and Trichuris trichiura*. This disagrees with the previous

studies carried out in Hoima where the most prevalent intestinal helminth was found to be Hookworm.

The higher prevalence of *Ascaris lumbricoides*in our finding maybe attributed to the low temperatures and high atmospheric humidity in our study area that favors development of *Ascaris lumbricoides* as compared to the higher temperatures of Hoima that mainly favors Hookworm development.

c) *Predisposing factors to intestinal Helminths infections*

The study revealed majority of study participants or pupils were not practicing anal cleaning and regular hand washing; only 28% of those who washed hands used soap and water; majority of participants 64.3% used plant leaves for anal cleaning yet their cleanliness is unknown. This was attributed to lack of toilet facilities like toilet papers and hand washing facilities in their respective schools. This puts these group at a greater risk of getting infected with intestinal Helminths. This is consistent with the study Matthys *et al*., (2011) that showed that washing hands without soap after defecation is a major risk factor to intestinal Helminths infections. This shows the need for health education and sensitization about transmission, prevention and control of intestinal helminth infections on addition to provision of hand washing facilities

Majority of pupils 498/798(62.4%) were drinking boiled water only at home and were getting water from unsafe sources which included ponds and stream where water quality is doubted hence putting community masses at risk of acquiring parasitic infections. This is in line with the study by Doni *et al.*, (2015) that showed that children who use drinking water from rivers, streams, springs, and wells had a significantly higher prevalence of intestinal parasitic infection. Unless these trend of risky practices are minimized by provision of reliable and safe sources of water for domestic using, parasitic infections will remain a health challenge in this area and in these group of people resulting into poor quality of life

V. Conclusion

The study findings revealed that Intestinal Helminths infections remains a health challenge among school going children in Kamuganguzi sub county; where *Ascaris lumbricoides and Trichuris trichiura* are the Helminths species responsible for infections despite the existing control and preventive interventions.

Poor anal cleaning practices and unreliable sources of water are the main predisposing factors for transmission and maintenance of Helminths infections in the study area.

VI. Recommendations

There is need to increase access to reliable and safe water sources to the society, intensify the current preventive and control interventions like deworming and community health sensitization regarding hygienic practices.

There is need to integrate anti helminths drug administration, water treatment and community sensitization programs in the country if parasitic infections are to be contained in this area.

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Authors' Contributions

MD, AKW, OLJ and AA conceptualized the idea, designed the study, data collection and analyzed the data, interpreted the data, and drafted the manuscript. KR played a supervisory role and mentorship where he guided in designing the study, proposal generation and reviewed the proposal, data analysis, report and prepared the manuscript, MD also assisted in mobilization of study participants, laboratory procedures and data collection.

Conflict of interest

All authors declare no conflict of interest in the study.

Submission declaration and verification

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Morphology and Pollen Studies of Three Varieties of *Impatiens Balsamina* Linn. (Balsalminaceae) in Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria

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Abstract- Pollen morphological studies were investigated among three varieties of *Impatiens balsamina* to delimit, classify and trace probable evolutionary relationships and also provide a general reference system into which other kinds of information can be incorporated. Qualitative and quantitative morphological studies were carried out on each variety of *Impatiens balsamina* at their reproductive stage. Pollen grains from fresh anthers of three varieties of *Impatiens balsamina* from Botany Department, Obafemi Awolowo University, Osun State, Nigeria were collected and acetolysed. The structural morphology of the pollen grains carried out with light microscope is reported. Characters measured on the pollen grains were pollen diameter, pollen wall thickness, furrow diameter and pore diameter. This was done with the aid of ocular micrometer.

Keywords: *Impatiens balsamina, pollen grains, evolution, morphology, acetolysis.*

GJSFR-C Classification: *FOR Code: 270499*

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Ojo. R. A. [α], Teniola, K. A. [σ] & Folorunso, A.E. [ρ]

Abstract- Pollen morphological studies were investigated among three varieties of *Impatiens balsamina* to delimit, classify and trace probable evolutionary relationships and also provide a general reference system into which other kinds of information can be incorporated. Qualitative and quantitative morphological studies were carried out on each variety of *Impatiens balsamina* at their reproductive stage. Pollen grains from fresh anthers of three varieties of *Impatiens balsamina* from Botany Department, Obafemi Awolowo University, Osun State, Nigeria were collected and acetolysed. The structural morphology of the pollen grains carried out with light microscope is reported. Characters measured on the pollen grains were pollen diameter, pollen wall thickness, furrow diameter and pore diameter. This was done with the aid of ocular micrometer. Number of pores per pollen and number of pollen per view was also counted. Pollen grain sizes of the varieties of *Impatiens balsamina* fall into one group which is Media (diameter 25-50μm). Acolpate, tetracolpate and pentacolpate pollen grains were encountered in all the three varieties of *Impatiens balsamina* studied. Tricolpate pollen was found only in red *Impatiens balsamina*. Hexacolpate pollen which was found in white and red varieties of *Impatiens balsamina* separates the red and white varieties from the pink variety. It is also a mark of evolutionary advancement over the pink variety.

Keywords: Impatiens balsamina, pollen grains, evolution, morphology, acetolysis.

I. Introduction

The genus *Impatiens* belongs to the order Ericales, family Balsalminaceae. There are 1,000 species mostly distributed in tropics and rarely in temperate regions (Mabberley, 2008) primarily in Asia and Africa, Europe and North America (Janssens *et al*., 2012). Species are territorial, no Africa species occurs naturally in Asia and no Asian species occur naturally in Africa (Christopher, 2013). The name *Impatiens* is derived from the fact that the seed capsule ejects from a flower when ripe. This action led botanists to suggest that the seeds were impatient to germinate (Christopher, 2013). *Impatiens balsamina* are annual herbs, and grow up to height of 60-100cm. *Impatiens balsamina* is widely cultivated as an ornamental for its attractive flowers. The flowers and leaves are often used across Asia for the traditional dyeing of a woman hair, nails, hands and feet (IPK Gatersleben, 2018). The stems, leaves and oil of the seeds are used in traditional Asian medicine for promoting blood circulation and for relieving pain and sore throats (Flora of China Editorial Committee, 2018; PROTA, 2018; USDA-ARS, 2018). The Chinese use *Impatiens balsamina* to counteract snakebites or the ingestion of poison fish. Juice extracted from the stem and added to rice liquor is known to reduce swelling and heal bruises. When dried, the stem may be pulverized and made into an ointment to relieve pain. Flowers are made into paste to treat back pain and neuralgia. The Vietnamese wash their hair with an extract of *I. balsamina* believing that it stimulates the follicles to grow hair.

Impatiens balsamina is cathartic, diuretic and emetic. The leaf juice is used as treatment against warts. The flowers are cooling, mucilaginous and tonic. The seed is expectorant and has been used in the treatment of cancer. The powdered seeds are given to women during labour in order to provide strength. A dye is obtained from the flowers and leaves for dyeing finger and toe nails. Regular ingestion of large quantities of *Impatiens* can be dangerous due to high mineral content especially for people with rheumatism, arthritis, gout and kidney stones.

Pollen characteristics have been used considerably in the taxonomy of angiosperms and can be applied in tracing the history of plant groups and species (Moore and Webb, 1978). Among some of the researchers who have worked extensively on the morphology of pollen grains and have emphasized the significance of pollen architecture in phylogeny are Patel and Datta (1958) and Sowunmi (1973). Palynological attributes of plants have attracted attention of many researchers in recent time (Adedeji, 2005; Arogundade and Adedeji, 2009). Nyananyo and Olowokudejo (1986) used seed coat morphology and palynological features of *Talinum* and *Calandrinia* to produce a more acceptable classification of the species in these taxa. Akinwusi and Illoh (1996) reported that palynology provides useful data for the taxonomy of the genus

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Hibiscus. Similarly, Azeez and Folorunso, (2014) on the phenology and pollen studies of some species of Annonaceae in Nigeria provide data for the evolutionary development in the genera of Annonaceae. Edeoga *et al*., (1996) and (1998) have utilized pollen attributes to establish probable evidence of relationships among certain groups of flowering plants in Nigeria. The main characters of taxonomic value in pollen grain are the number and position of apertures (colpi and pores), pollen wall morphology and sizes of pollen grains.

Except for the global pollen project, which is not even complete, there is no reported work on the pollen morphology of *Impatiens balsamina*. The aim of this study is therefore to use the characters of pollen of the three varieties (pink, red and white) of *Impatiens balsamina* to delimit, classify and trace evolutionary relationships among the three varieties of *Impatiens balsamina* in Nigeria.

II. Materials and Methods

Collection of Pollen grains

Pollen grains in anthers were collected every day for two weeks from mature plants of the three varieties (red petal, pink petal and white petal) of *Impatiens balsamina*. The collection sites are given in table 1. The collected pollen grains were stored in 70% ethanol before acetolysis was carried out.

Table 1: Collection sites for the varieties of *Impatiens balsamina* L. studied

Species	Location	GPS
Impatiens balsamina (Red petal)	Department of Botany, Obafemi Awolowo University, Ile Ife	7^0 31´8´´N $4^0$31´34´´E
Impatiens balsamina (White petal)	Department of Botany, Obafemi Awolowo University, Ile Ife	7^0 31´8´´N $4^0$31´34´´E
Impatiens balsamina (Pink petal)	Department of Botany, Obafemi Awolowo University, Ile Ife	7^0 31´8´´N $4^0$31´34´´E

The collected pollen grains were acetolysed according to the procedure of Erdtman (1960), with slight modifications;

The preserved pollen grains were macerated with stirring rod inside the vials to separate it into particles. The macerated pollen grains were poured into centrifuge tubes which were labelled A, B, C. Equal volume of alcohol was poured into each centrifuge tube. The samples were then arranged into the centrifuge facing each other. The specimens were centrifuged at 3,500rpm for 20 mins. After this, the ethanol was poured off, 5ml glacial acetic acid was added and the specimen was stirred properly with stirring rod. The sample was centrifuged again at 3,500rpm for 20 mins. The glacial acetic acid was poured off. Acetolysis mixture (sulphuric acid and acetic anhydride) was prepared in the ratio 1:6. 30ml of H_2S0_4 and 180ml of acetic anhydride was prepared and added to the sediment. Water was put in a beaker and heated on hot plate, the temperature was checked at intervals, on reaching 70^0C, the test tubes were placed inside the beaker and allowed to boil to 100^0C as the samples were boiling, they were being stirred. The samples were allowed to boil and bring out bubbles before the beaker was removed from the hot plate. The samples were removed from the test tubes and put inside wooden test tube holder and allowed to cool. The samples were placed inside the centrifuge again and centrifuged at 3,500rpm for 25 mins. The acetolysis mixture was decanted. 10ml glacial acetic acid was added to the sediment and stirred with stirring rod. The samples were centrifuged again at 3,500rpm for 25 mins. The glacial acetic acid was decanted. Distilled water was added and shaken well. The samples were centrifuged again at 3,500rpm for 25 mins. and decanted. The acetolysed pollen grains were stored in vials. 2ml dilute glycerine was added to the acetolysed pollen grains stored in vials.

Acetolysed pollen grains in dilute glycerine were properly examined under the light microscope. 20 pollen grains chosen at random were studied per variety. Measurements of the diameter of the pollen grains, furrow diameter and pollen wall thickness were taken for each variety with the aid of an ocular micrometer inserted in the eyepiece of the microscope. The measurements were later multiplied by an ocular constant with respect to power under which they were taken in order to convert them to micrometer. Other qualitative characters studied on the pollen grains are the shape of the pollen, the number of the furrows on the pollen wall and the colour of the pollen grains.

Photomicrographs of the acetolysed pollen grains were taken using Amscope MT microscope camera version 3.001 attached to a light microscope. Pollen structure description was according to Moore and Webb (1978).

The mean of the data generated from this work was calculated. Statistical analysis for significance differences among the three varieties was carried out. A table illustrating the mean of the pollen characters measured was prepared and Cluster diagram of the values was also made using Paleontological Statistics Software Package (PAST). Duncan Multiple Range Test (DMRT) was also done using Statistical Package for the Social Sciences (SPSS) to determine if the means are significantly different.

III. Results

The pictures of the varieties of *Impatiens balsamina* L. studied are as shown in Plate 1.

At reproductive stage, the following morphological studies were carried out on each variety of *Impatiens balsamina* L. for all identifiable character difference and similarities as shown in Tables 2 and 3 respectively.

A B C

A: Red *Impatiens balsamina*; B: White *Impatiens balsamina*; C: Red *Impatiens balsamina*

Plate 1: Pictures of the varieties of *Impatiens balsamina* L. studied

Table 2: Qualitative Morphological Characters of the Varieties of *Impatiens balsamina* L

Characters	Red	White	Pink
Leaf shape	Lanceolate	Lanceolate	Lanceolate
Leaf apex	Acuminate	Acute	Acuminate
Leaf margin	Serrate	Serrate	Serrate
Leaf texture	Glabrous	Slightly pubescent	Slightly pubescent
Leaf base	Cuneate	Acuminate	Cuneate
Leaf arrangement	Alternate	Alternate	Alternate
Venation pattern	Pinnately net-veined	Pinnately net-veined	Pinnately net-veined
Stipule	Absent	Absent	Absent
Petiole	Absent	Absent	Absent
Stem color	Red	Light green	Red
Fruit color	Light green	Light green	Light green
Fruit stalk color	Red	Light green	Red
Pedicel color	Red	Light green	Red
Anther color	White	White	White
Filament color	Red	White	Light red

Table 3: Quantitative Morphological Characters of the varieties of *Impatiens balsamina* L

Characters	Red	White	Pink
Leaf length (cm)	10.41	10.28	10.16
Leaf width (cm)	1.74	1.57	1.79
Internode distance (cm)	1.74	1.36	1.60
Number of leaf per stem	82.00	61.00	80.00
Plant height from soil (cm)	69.71	60.50	57.31
Petal width (cm)	3.31	2.40	2.59
Length of pedicel (cm)	1.60	1.47	1.75
Length of fruit stalk (cm)	1.80	1.97	1.89

Table 4: Analysis of Quantitative morphological characters of the three varieties of *Impatiens balsamina* L. studied

Parameters	Red	White	Pink
Number of leaves per stem	81.60 ± 5.97[a]	61.00 ± 8.73[b]	79.90 ± 5.01[ab]
Plant height from soil	69.71 ±2.04[a]	60.50 ± 1.83[b]	57.31 ± 2.05[b]
Leaf length (cm)	10.41 ± 0.29[a]	10.28 ± 0.33[a]	10.16 ± 0.39[a]
Leaf width (cm)	1.74 ± 0.05[a]	1.57 ± 0.08[a]	1.79 ± 0.10[a]
Petal width (cm)	3.31 ± 0.14[a]	2.40 ± 0.05[b]	2.59 ± 0.08[b]
Length of pedicel (cm)	1.60 ± 0.05[ab]	1.47 ± 0.05[b]	1.75 ± 0.06[a]
Internode distance (cm)	1.74 ± 0.13[ab]	1.36 ± 0.10[b]	1.60 ± 0.11[a]
Length of fruit stalk (cm)	1.80 ± 0.08[a]	1.97 ± 0.10[a]	1.89 ± 0.05[a]

** Means with the same alphabet superscript across the row are not significantly different at P ≤ 0.05.

This study reveals that the three varieties of *Impatiens balsamina* L. are erect herbs. All the three varieties are sessile and exstipulate leaves which are alternately arranged. The leaves of all the three varieties are pinnately net-veined, have serrate leaf margin and lanceolate leaf shape. The leaf base of red and pink *Impatiens balsamina* is cuneate while that of white *Impatiens balsamina* is acuminate. The leaf apex of red and pink *Impatiens balsamina* is acuminate while that of white *Impatiens balsamina* is acute. The leaf texture of white and pink *Impatiens balsamina* is slightly pubescent while that of red *Impatiens balsamina* is glabrous. The stem colour of red and pink *Impatiens balsamina* is red, while that of white *Impatiens balsamina* is light green. The pedicel of red and pink *Impatiens balsamina* is red in colour while that of white *Impatiens balsamina* is light green in colour. The anther colour of the three varieties is white. The filament colour of red *Impatiens balsamina* is red, that of white is white and that of pink is light red. The fruit colour of the three varieties is light green. The fruit stalk colour of red and pink *Impatiens balsamina* is red while that of white *Impatiens balsamina* is light green.

Generally, the shape of the pollen grains ranged from oval, to round, to rectangular, pentagonal shapes, with only hexagonal shape in white and red varieties (Plates 1-3). The two types of aperture, the colpi and the pore were present in the three varieties. Colpi were fissure-like or slit-like apertures, also known as furrows, while pores were reticulate. The pollen grains are light brown in colour.

Red Impatiens balsamina

The pollen types present were acolpate (Plate 2A), tricolpate (Plate 2B), tetracolpate (Plate 2C), pentacolpate (Plate 2D) and hexacolpate (Plate 2E). The mean diameter of the pollen grains was 33±0.46μm. The colpi were situated in the polar region only, while pores were found in the equatorial region of the pollen grains.

White Impatiens balsamina

The pollen types present were acolpate (Plate 3A), tetracolpate (Plate 3B), pentacolpate (Plate 3C) and hexacolpate (Plate 3D). The mean diameter of the pollen grains was 31.80±0.72μm. The colpi were situated in the polar region only, while pores were found in the equatorial region of the pollen grains.

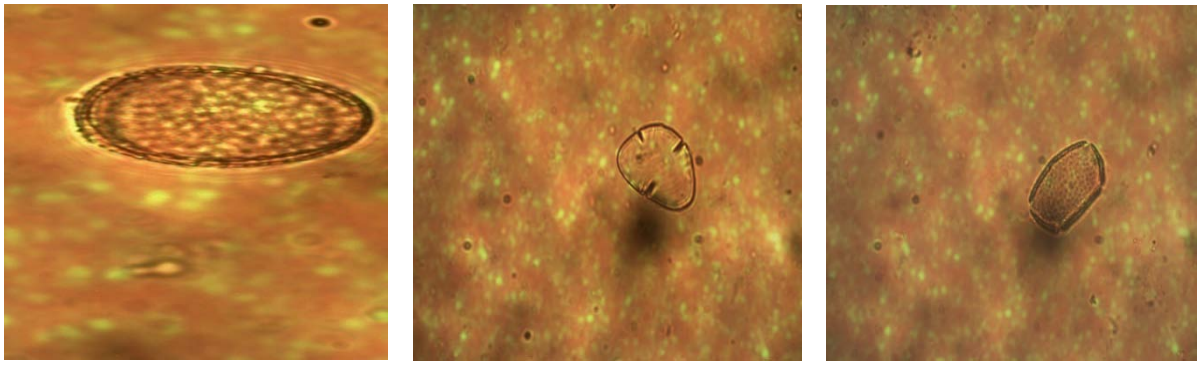
Pink Impatiens balsamina

The pollen types present were acolpate (Plate 4A), tetracolpate (Plate 4B) and pentacolpate (Plate 4C). The mean diameter of the pollen grains was 30.50±0.18μm. The colpi were situated in the polar region only, while pores were found in the equatorial region of the pollen grains.

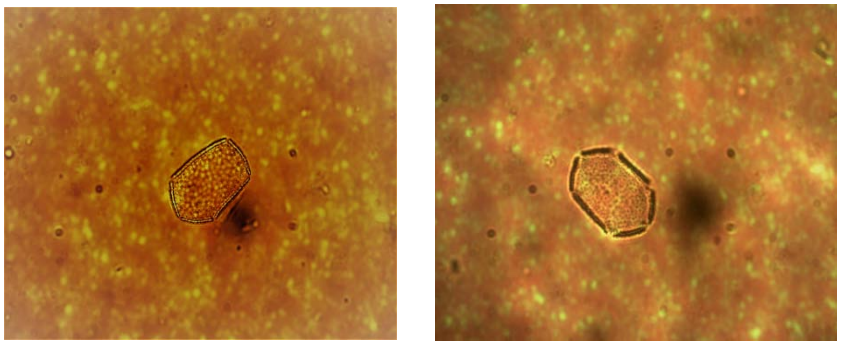
Table 5: Quantitative characters of the three varieties of *Impatiens balsamina* L. studied based on the pollen grain attributes

Parameters	Red	White	Pink
Pollen diameter(um)	33.00 ± 0.46[a]	31.80 ± 0.72[a]	30.50 ± 0.18[a]
Pollen wall thickness(um)	2.20 ± 0.06[a]	2.10 ± 0.05[a]	2.30 ± 0.03[a]
Furrow diameter(um)	2.10 ± 0.08[a]	3.75 ± 0.34[a]	2.25 ± 0.08[a]
Number of colpi	4.45 ± 0.2[a]	4.00 ± 0.32[a]	3.50 ± 0.40[a]
Number of pollen grains per view	5.80 ± 0.63[a]	2.60 ± 0.28[b]	5.80 ± 0.50[a]
Pore diameter	3.25 ± 0.20[a]	3.87 ± 0.09[a]	6.87 ± 0.59[b]
Number of pores	92.4 ± 9.51[a]	103.1 ± 8.13[b]	72.4 ± 5.47[ab]

** Means with the same alphabet superscript across the row are not significantly different at P ≤ 0.05.



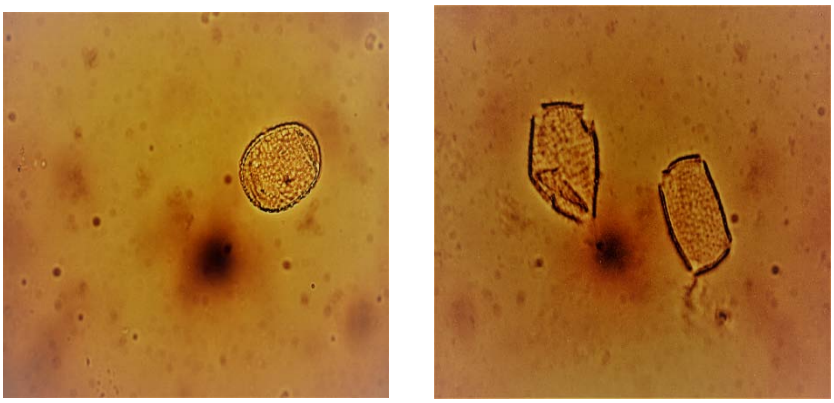
A B C



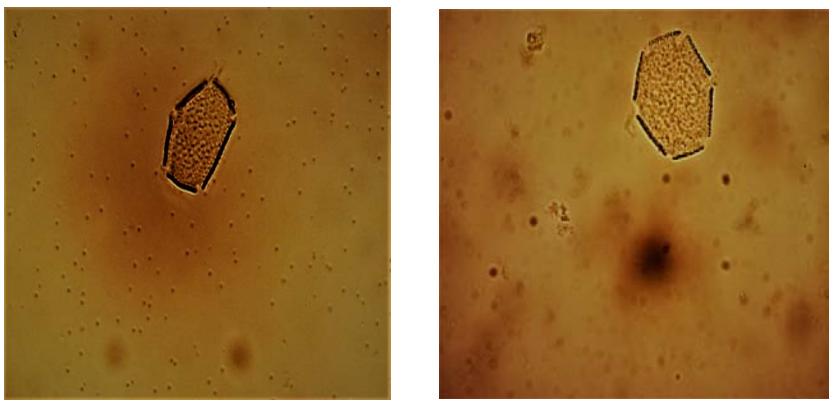
D E

A: Acoplate; B: Tricolpate; C: Tetracolpate; D: Pentacolpate; E: Hexacolpate

Plate 2: Pollen grains of Red *Impatiens balsamina*



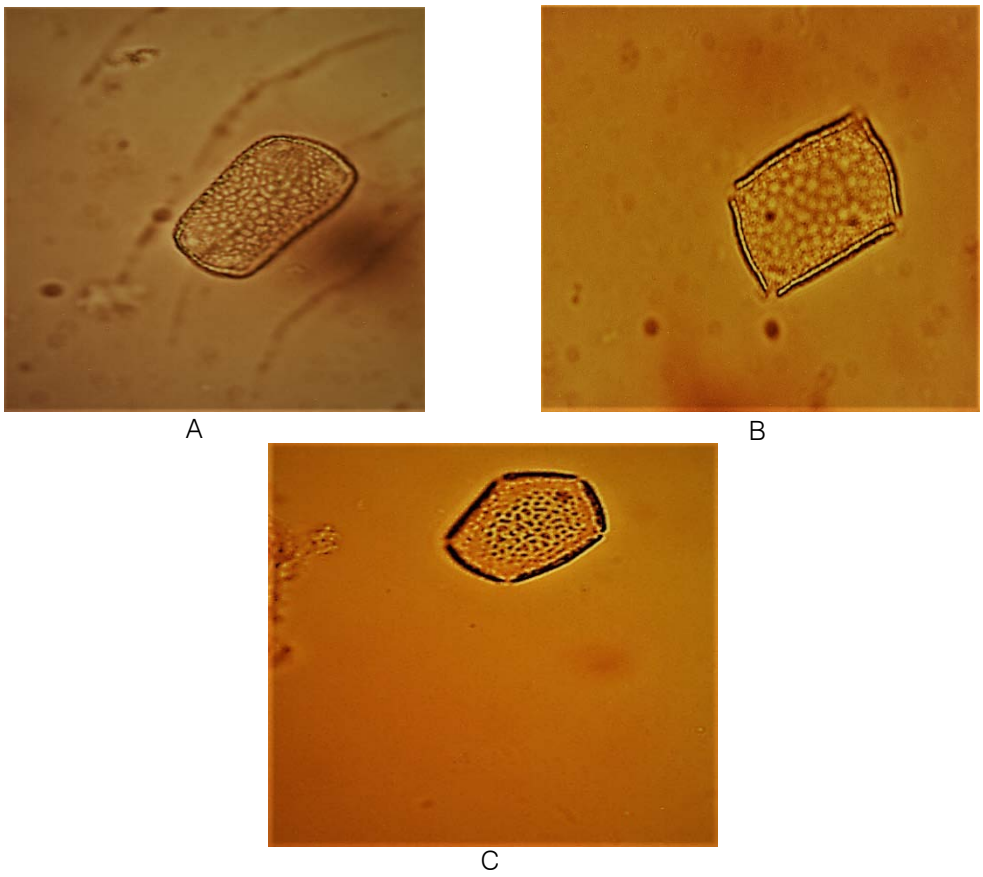
A B



C D

A: Acolpate; B: Tetracolpate; C: Pentacolpate; D: Hexacolpate

Plate3: Pollen grains of White *Impatiens balsamina*



A: Acolpate; B: Tetracolpate; C: Pentacolpate

Plate 4: Pollen grains of Pink *Impatiens balsamina*

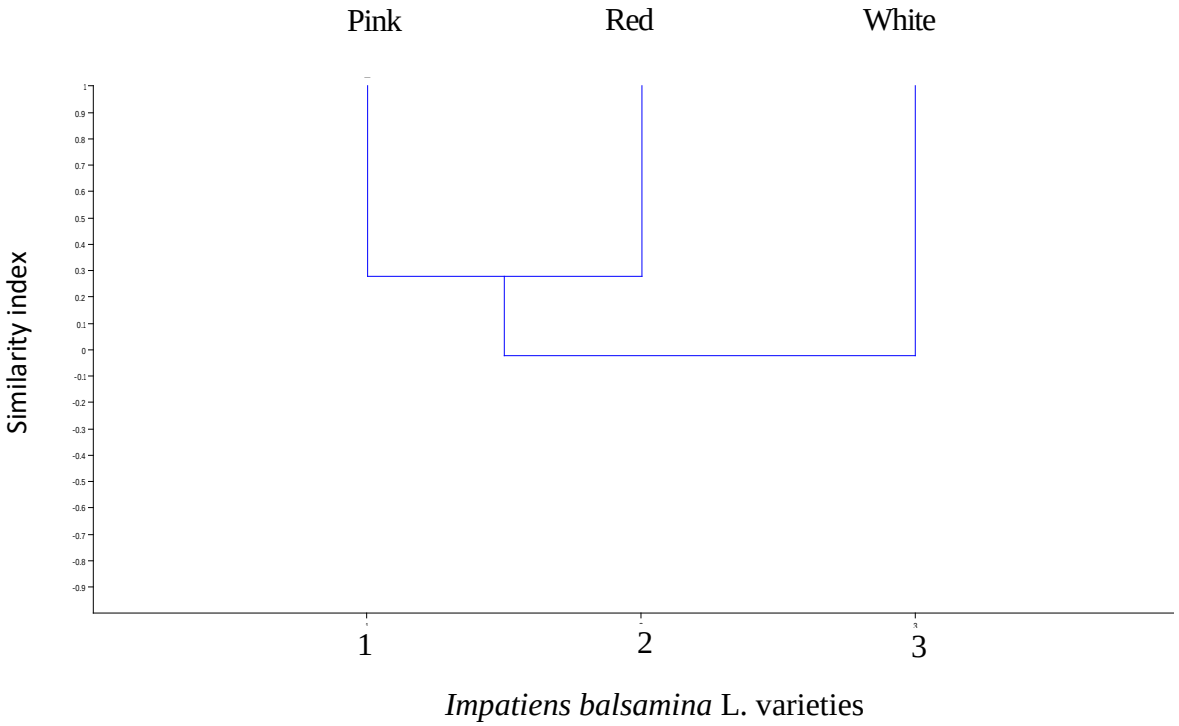


Figure 1: Dendrogram of the pollen grain attributes and morphological characters of the three varieties of *Impatiens balsamina* L. based on their quantitative and qualitative characters studied.

IV. Discussion

The pollen grains of the varieties of *Impatiens balsamina* L. studied are similar morphologically though there were some slight differences among them especially in white *Impatiens balsamina* which has the largest furrow diameter and has less number of pollen grains per view. There was also variation in the pollen shapes which ranged from oval to round to rectangular and pentagonal shapes in all the varieties with hexagonal shape only in white and red varieties.

The acolpate, tetracolpate and pentacolpate pollen grains were encountered in all the varieties studied. These three pollen types can therefore be affirmed as generic. Tricolpate pollen was found only in red *Impatiens balsamina*. Hexacolpate pollen was found in white and red varieties only. This separates the white and red varieties from the pink variety. It is also a mark of evolutionary advancement over the pink variety. Various researchers have separated species of plants based on the number of colpi on their pollens (Akinwusi and Illoh, 1996; Adedeji, 2005; Arogundade and Adedeji, 2009). Number of colpi on pollen grains has been a useful tool in tracing evolutionary relationship among the species of a genus. The advanced dicotyledons have more colpi than the primitive ones, with either a colpus (monocolpate) or none at all (acolpate) (Walker, 1976; Adedeji, 2005).

The shape of the pollen grains can also be employed in separating the varieties of *Impatiens balsamina* in this study. Oval, round, rectangular and pentagonal shapes were encountered in the three varieties of *Impatiens balsamina* studied, while hexagonal shape was found only in the red and white varieties of *Impatiens balsamina*. Arogundade and Adedeji, (2009) also separated some species of genus *Ocimum* based on their shape.

White *Impatiens balsamina* has the largest furrow diameter and the smallest number of pollen grains per view. This further separates white variety from red and pink varieties. The result of Duncan Multiple Range Test (DMRT) for means separation reveals that the pollen diameter, pollen wall thickness, furrow diameter and number of colpi of the three varieties of *Impatiens balsamina* are not significantly different from each other. Also, the number of pollen grains per view of white *Impatiens balsamina* is significantly different from that of red and white varieties of *Impatiens balsamina*.

Pollen grains have been classified into groups according to their size by Erdtman (1952) as Perminuta (diameter less than 10μm), Minuta (diameter 10-25μm), Media (diameter 25-50μm), Magna (diameter 50-100μm), Permagna (diameter 100-200μm) and Giganta (diameter greater than 200μm). Based on this classification, the pollen grain of the varieties of *Impatiens balsamina* could be described as media (diameter 25-50μm). In a similar study, Akinwusi and Illoh (1996) used pollen grain size to group some species of *Hibiscus* in Nigeria.

From the result of the cluster analysis, the dendrogram revealed that red and pink *Impatiens balsamina* are more closely related since they are clustered together at the same similarity level of 0.3, while white *Impatiens balsamina* stood out on its own.

In conclusion, this study revealed that pollen morphology provides data for the recent evolutionary development among the three varieties of *Impatiens balsamina* L. studied. Also, the presence of hexacolpate pollen grains in red and white varieties is a mark of evolutionary advancement among the three varieties of *Impatiens balsamina* L. studied. It also revealed the morphological characters that distinguished these three closely related varieties from one another.

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Fellows/Associates of Science Frontier Research Council

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19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

Criterion for Grading a Research Paper (Compilation) by Global Journals

Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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