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Biological Sciences

Cassava Plant Diseases

Endophytes Improve Survival

Highlights

Broiler Chicks in an Outbreak

Intestinal Helminthiasis in Primary

Discovering Thoughts, Inventing Future

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The Difference between Life and Death: Fungal Endophytes Improve Survival and Increase Biomass in Multiply-Stressed Barley

By Brian R. Murphy, Lucia Martin Nieto, Fiona M. Doohan & Trevor R. Hodkinson
Trinity College Dublin, Ireland

Abstract- Sustainable farming systems are required to allow crops to better cope with the simultaneous multiple stresses that they grow under or are likely to be exposed to under future climate change. Fungal endophytes could form part of the solution. They have been shown to improve important agronomic traits under a single stress, but few studies have investigated the impact of endophytes on growth or disease resistance when exposed to multiple stresses. We compared the performance of the barley cultivar Propino when inoculated with five fungal root endophytes, either individually or combined, derived from wall barley (Hordeum murinum) and grown in optimal conditions (OC) and under a combined drought, heat, nutrient and pathogen stress (MS). We found a greater endophyte-induced improvement in important agronomic traits in the MS plants compared with the OC plants. For the MS plants only 13% of the controls survived to the end of the experiment compared with 80% of the endophyte treatments.

Keywords: *barley, fungal root endophytes, multiple stresses, agronomic traits, climate change.*

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The Difference between Life and Death: Fungal Endophytes Improve Survival and Increase Biomass in Multiply-Stressed Barley

Brian R. Murphy [α], Lucia Martin Nieto [σ], Fiona M. Doohan [ρ] & Trevor R. Hodkinson [ω]

Abstract- Sustainable farming systems are required to allow crops to better cope with the simultaneous multiple stresses that they grow under or are likely to be exposed to under future climate change. Fungal endophytes could form part of the solution. They have been shown to improve important agronomic traits under a single stress, but few studies have investigated the impact of endophytes on growth or disease resistance when exposed to multiple stresses. We compared the performance of the barley cultivar Propino when inoculated with five fungal root endophytes, either individually or combined, derived from wall barley (*Hordeum murinum*) and grown in optimal conditions (OC) and under a combined drought, heat, nutrient and pathogen stress (MS). We found a greater endophyte-induced improvement in important agronomic traits in the MS plants compared with the OC plants. For the MS plants only 13% of the controls survived to the end of the experiment compared with 80% of the endophyte treatments. In MS plants, the endophytes induced increases in the number of tillers and root and shoot biomass. The improvements were most significant for barley inoculated with a combination of all five endophytes. These results demonstrate potential for these endophytes as barley inoculants in similarly multiply-stressed farming environments. To our knowledge, this is the first experiment which has examined the effect of inoculating endophytes from a congeneric wild relative of barley onto abiotically and biotically stressed barley.

Keywords: *barley, fungal root endophytes, multiple stresses, agronomic traits, climate change.*

I. Introduction

Biotic and abiotic stresses such as extreme temperatures, low water availability, low nutrient availability and pathogenic infections are frequently simultaneously encountered by plants in both natural and agricultural systems(Langridge *et al.* 2006). For example, high temperature and water stress are often co-associated. Abiotic stresses alone are estimated to reduce global crop yields by over a half of that possible under optimal growing conditions (Boyer 1982). Abiotic stresses, in particular, may increase in the future due to global climate change (IPCC 2014) and predicted increases in drought and temperature-related stresses are expected to reduce crop productivity even further (Ciais *et al.* 2005; Larson, 2013). In order to successfully address the challenge of future food security it is necessary to increase yields, find more sustainable farming methodologies and to cultivate additional farmland. Potential exists for further extending farming on to marginal, arid, and semi-arid lands, especially in the developing world (Lantican *et al.*, 2003).The key risk associated with the likelihood of an increase in multiply stressed growing conditions will be reduced crop productivity, with strong adverse effects on regional, national, and household livelihood and food security (IPCC 2014).

These risks will be exacerbated by the exponential growth in the world population(Coleman-Derr & Tringe 2014), and will be most significant for the important global crops, including barley. Barley is grown on 56 Mha worldwide with a 2005 – 2008 mean production of 1.43×10^{11} kg(Newton *et al.* 2011), and while it can be grown profitably on marginal land, future increases in multiple stressors will require new crop varieties and new farming techniques to maintain acceptable crop yields.

Traditional approaches to breeding crop plants with improved stress tolerance have made some progress and wild relatives and landraces of cereal crops still offer great potential for breeding desired traits into crops(Langridge *et al.* 2006).However, conventional breeding practices often neglect the complex ecological context of the soil environment in which the crop is grown(Coleman-Derr & Tringe 2014), and other supplementary techniques are needed to improve barley stress tolerance. A class of microorganisms called endophytes have been shown to enhance biotic and abiotic stress tolerance in plants(Baltrusch at *et al.* 2008; Worchel *et al.* 2012; Hubbard *et al.* 2013; Murphy *et al.* 2014a). Endophytes are microorganisms (bacteria, fungi and unicellular eukaryotes) which can live at least part of their life cycle inter- or intra cellularly inside of plants usually without inducing pathogenic symptoms. This

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can include competent, facultative, obligate, opportunistic and passenger endophytes. Endophytes can have several functions and/or may change function during their lifecycle (Murphy *et al.* 2014a).

The complexities of stress responses essentially limit the predictive relevance of experimental evidence using individual stresses, suggesting that combinatorial studies of stress responses may be the best approach (Mittler 2006; Atkinson & Urwin 2012). For example, with heat stress alone plants can cool their leaves by transpiration, but if heat stress is combined with drought, plants cannot open their stomata to cool their leaves, leading to overheating (Rizhsky *et al.* 2002). Plants activate a specific and unique stress response when subjected to a combination of multiple stresses (Rizhsky *et al.*, 2004), so current techniques for developing and testing stress-tolerant plants by imposing each stress individually may be inadequate (Mittler & Blumwald, 2010). Signalling pathways associated with combinations of biotic and abiotic stresses may act antagonistically, changing the plant response in ways not predictable from individual stresses (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008).

While previous studies have examined the effects of one or two simultaneous stresses on barley, we aimed to test the effects of inoculating fungal root endophytes (hereafter endophytes) derived from a wild barley species, *Hordeum murinum* subsp. *murinum* L.,onto a barley cultivar growing under a combination of heat, drought, pathogen (*Gaeumannomyces graminis* var. *tritici*) and nutrient stress. *Hordeum murinum* is an annual grass and a ruderal of roadsides, rough grassland and waste places (Streeter *et al.* 2009; Stace 2010). As the species generally grows in abiotically-stressed environments (El-Shatnawi *et al.* 1999; Myrna Johnston *et al.* 2009; Murphy *et al.* 2014a), it may have evolved symbiotically-conferred stress tolerance associated with endophyte infection (Rodriguez *et al.* 2008). Endophytes isolated from *H. murinum* may have the potential to benefit cultivated barley in similar stressed conditions.

II. Materials and Methods

Five endophyte isolates - 0401IA76 (GenBank ID: KM492846), 0406050(2)A (GenBank ID: KM492844), 0406050(2)C (GenBank ID: KM492845), 040901(3) (GenBank ID: KM492837) and 040906(4) (GenBank ID: KM492839) - were selected from a previous experiment which characterised endophytes that were isolated from wild populations of *Hordeum murinum* in Ireland (Murphy *et al.* 2014a). The provided nuclear ribosomal internal transcribed sequences (nrITS) DNA sequences for these strains were compared with existing GenBank accessions to establish the identity of the strains.

Untreated seeds of the barley cultivar 'Propino' (Goldcrop Seeds, Cork, Ireland) were used. Seeds were surface-sterilised by soaking in 5% NaClO for 15 min, rinsing three times with 70% ethanol and then rinsing five times with pure water. The growth compost of John Innes No.3 formulation (Westland Horticulture Limited) was placed into 1.5 litre washed and sterilised (soaked for 2 hours in 5% NaClO then rinsed × 5 with tap water) plastic pots. For each of the seven inoculation treatments (including a control), twenty five seeds of barley, in 5 pots containing 5 seeds each, were sown at 30 mm depth and either inoculated with an inoculant solution of 250μl containing one of the five endophytes or an inoculant solution of 250μl containing a combination of all five endophytes (AllEndos). The inoculant solution was prepared by mixing 10 mg of each fungal culture with 8 ml of pure water and stirring with a magnetic bar for 2 mins at 25°C. 250μl of the solution was directly inoculated onto each seed. For the controls, the seeds were inoculated with 250μl pure water. Every seed was also directly inoculated with a 250ul solution of the common and serious barley pathogen *Gaeumannomyces graminis* var.*tritici* ("take-all") (Gen Bank Accession KF018415), prepared as above.

Pots were placed into a controlled environment chamber, then randomly relabelled with a single number by a third party, to produce a double-blind and randomised setup. The pots were moved to a new position within the growth chamber every 3 days. The environmental settings of the growth cabinet (Conviron PGR14) were programmed to produce a 14 hr photoperiod at a compost surface illumination of 220 μmol.m^{-2} s^{-1}, a photoperiod temperature of 33°C reducing to 12°C in the dark period and a photoperiod relative humidity of 45%, increasing to 65% in the dark.

Three covered culture dishes containing five sterilised barley seeds on malt extract agar (Fluka 38954) were kept in the growth chamber during the experimental period to test for seed surface sterilisation success and to monitor any contamination that may be present from seed-produced fungal infection.

The seedlings were thinned to three plants per pot, 12 days after germination, producing 15 individual plant replicates for each treatment. A Germination Index (GI) was calculated using the formula:

$$GI = ((G_t / G_n) / G_n)$$

where G_t is the cumulative number of days to germination for all seeds and G_n the total number of seeds germinated. Soil moisture content at a depth of 50 mm was measured daily using a Delta-T Devices HH2 WET sensor kit (Delta-T Devices, Cambridge, UK) and pots were watered with tap water only when the soil moisture content was between 10% and 15% which was when the barley plants were starting to wilt and showed a drought-associated colour change. The pots were watered until soil moisture content was at field capacity (~45%). Total water input was 4.19 litres per pot. All

pots were given a liquid fertiliser (Bayer Phostrogen®) at each watering. Total nutrient input per plant was: ammoniacal N = 4mg, ureic N = 20mg, Total N = 24mg, P = 20mg, K = 40mg, Mg = 4mg, S = 8mg, Ca = 4mg and traces of Boron, Copper, Iron, Manganese, Molybdenum and Zinc. Inputs for nitrogen (N), phosphorous (P) and potassium (K) were approximately 6%, 17% and 16% respectively of that recommended for spring barley growing on low-nutrient soils (http://www.teagasc.ie/crops/winter/fertilisers/winter_cereals_fertiliser_requirements.pdf) so plants were severely nutrient-stressed. A liquid fertiliser was used because the low water input may have resulted in incomplete nutrient delivery if a solid fertiliser formulation were used.

A further set of 25 plants for each treatment were grown in close to optimal conditions (OC), with the environmental settings programmed to produce a 14 hr photoperiod at a temperature of 21°C reducing to 12°C in the dark period and a constant 70% relative humidity. The seedlings were thinned to three plants per pot, 12 days after germination, producing 15 individual plant replicates for each treatment. A Germination Index (GI) was calculated as above. Plants were watered to maintain the compost at near field capacity and total water input was 6.39 litres per pot. Nutrient input per plant was approximately five times that of the stressed plants (ammoniacal N = 20mg, ureic N = 100mg, Total N = 120mg, P = 90mg, K = 220mg, Mg = 20mg, S = 40mg, Ca = 20mg and traces of Boron, Copper, Iron, Manganese, Molybdenum and Zinc).

The number of days to reach selected Zadoks stages (Zadoks *et al.* 1974) was recorded for each plant. Plants were grown for 90 days (13 weeks) from date of sowing, then harvested and processed in one day. Before processing the plants, four 5 mm pieces of mid-section root from each plant were surface-sterilised and incubated on half-strength malt extract agar at 25°C in the dark to test for endophyte presence. Endophyte emergence was recorded over the next 35 days, and emergents were identified by morphological examination and by sequencing of the internal transcribed region (ITS) of nuclear ribosomal DNA(nrDNA). For the DNA analysis, 20 mg of fungal material was scraped from the agar surface and placed into shaker tubes. DNA was extracted using a Qiagen DNeasy mini kit, following the Qiagen protocol, producing 200 µl of DNA extract for each isolate. PCR was carried out on the DNA extracts using the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) primers ITS1 and ITS4 (White *et al.* 1990). The thermal cycling parameters were programmed to optimise primer annealing, consisting of: 3 min at 95°C; 9 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C; 20 cycles of 30 sec at 94°C, 1 min at 56°C, 3 min at 72°C; a final extension for 7 min at 72°C. PCR products were cleaned up using Exonuclease (New England Biolabs) and Shrimp Alkaline Phosphatase (ExoSAP ; Roche). Purified PCR products underwent cycle sequencing using the reverse ITS4 primer (4 pmol) or forward ITS1 primer (4 pmol) in separate reactions with the ABI BigDye 3.1 kit (Foster City, CA). The products were further purified using a BigDye XTerminator purification kit and protocol. DNA was sequenced using an Applied Bio systems 3130xL Genetic Analyzer. The recovered sequences from the roots of each treatment were compared to the sequences of the original inoculants.

Pots were selected for processing in random order. Measurements were made for each plant of fresh and dry weights of shoots and roots, mean height of plants to tip of highest leaf and number of tillers. Shoot and root tissue from each plant of the MS treatment was inspected for signs of *Gaeumannomyces graminis* var. *tritici* infection and the degree of infection estimated as a proportion of total tissue showing signs of disease. All plant parts were separately dried in ovens for 7 days at 65°C before dry weights were measured.

Data analysis was carried out using single and two-factor ANOVA with Bonferroni correction and Pearson's correlation statistical analyses supplied with Data desk® 6.1.

III. Results

When we compared the provided nrITS sequences of the endophyte strains with GenBank accessions, we found that they were only distantly related to known fungi (Table 1) with an overall mean pairwise similarity of only 88%, and thus represent relatively novel organisms. It would therefore be unwise to assign these strains to a particular taxon, and we will refer to them throughout using the strain codes.

While two of the multiply-stressed (MS) endophyte treatments, 040605(2)A and 040605(2)C, had a greater germination index (GI) than the control ($P < 0.01$), there was no overall difference in germination index between endophyte treatments and control. We found large and significant differences between optimally grown plants (OC) and plants subjected to multiple stresses (MS) (Table 2). The main difference was that all of the OC control and treatment plants survived until the end of the experiment, whereas only 13% of MS control plants survived. However, over 80% of the endophyte-inoculated MS plants survived. For two of the MS endophyte treatments, 040906(4) and 040605(2)A, all of the plants survived. Although all of the OC plants produced seeds, only 10% of MS plants produced stems with rudimentary flowering structures, and none of these produced any heads with grains. Most measured barley traits showed greater values for the OC plants (Figure 1); the mean height of OC plants was twice that of the MS plants; the mean number of tillers for the OC plants was five times greater than the MS and the mean shoot dry weight for the OC plants was over three times greater than the MS. However, the

mean root dry weight was exactly the same for both OC and MS plants. The root dry weight for the control plants was greater than all endophyte-inoculated plants in the OC treatment, whereas in the MS plants the root dry weight for all endophyte-inoculated plants was greater than the control. Final overall comparisons between control and endophyte inoculated plants revealed a significant overall improvement over the control in agronomic barley traits for the MS plants ($P < 0.01$) but with no detectable differences for the OC plants.

For the MS plants that survived until the end of the experiment, we found significant differences in trait performance between control and endophyte-inoculated plants (Figure 2 and Figure 3).The mean root dry weight differed significantly between treatments (single factor ANOVA, $F_{6,98} = 8.32$, $P < 0.001$), where all of the endophyte treatments had greater root dry weight than the control ($P < 0.001$). The mean plant height, shoot dry weight and number of tillers for the endophyte treatments were all significantly greater than the control ($P < 0.05$), with one exception: there was no detectable difference in shoot dry weight and number of tillers between the control and the endophyte treatment 040901(3). The combined endophyte inoculant (AllEndos) was the treatment that gave the greatest improvement for all harvest traits (number of dead plants, plant height, number of tillers, shoot dry weight, root dry weight) in the multiply-stressed plants ($P < 0.01$ for every trait).

We found no difference between treatments in the MS plants for the proportion of root and shoot tissue displaying signs of *Gaeumannomyces graminis* var. *tritici* infection, where all plants had less than 5% of total root tissue with disease symptoms, but with no visible symptoms on above ground tissue.

At the end of the experiment, the three covered culture dishes containing five sterilised barley seeds on malt extract agar (Fluka 38954) that were kept in the growth chamber during the experimental period produced no evidence of seed-produced fungal infection.

A mean 50% (182) of root pieces that were removed from the MS plants at harvest produced fungal endophyte emergents. When we compared the emergent endophyte ITS sequences with the original inoculants, we found that each of the recovered sequences exactly matched that of the original.

IV. Discussion

Crop growers have long known that it is often the simultaneous occurrence of multiple stresses, rather than a particular stress condition, that is most lethal to crops (Mittler 2006).In this study, we have shown that endophytes derived from a wild relative of barley can reduce the lethal effect of a combination of heat, drought, nutrient and pathogen stress. In fact, very few of the control plants survived to the end of the experiment, and those that did survive performed significantly worse than the endophyte-inoculated plants. While each individual endophyte treatment induced improvements in several agronomic traits, it was the combined endophyte inoculant that improved all barley traits in the multiply-stressed plants most significantly. This suggests that a combination of endophytes may give the best results in a more realistic agricultural environment where the interactions between many competing microorganisms can make the outcome from a single endophyte inoculant uncertain.To our knowledge, this is the first ever study of the effect of endophytes derived from a congeneric wild relative of barley grown under multiple stresses.

An overall analysis of barley traits showed that there was a neutral effect due to the endophyte treatments in the optimally grown plants (OC) compared with a highly significant improvement in all traits for the multiply-stressed plants (MS). This suggests that barley plants may derive the most benefit from endophyte infection in stressful growing conditions (Singh *et al.* 2011; Khan *et al.* 2013; Song *et al.* 2014). However, not all of the measured traits showed improvements induced by endophyte inoculation under both regimes. In particular, root dry weight for the OC plants was significantly higher in the control plants, whereas root dry weight was lower for controls in the MS plants, suggesting that the endophyte infection stimulates greater root activity under the multiple stresses applied here. There have been contradictory reports regarding the relationship between endophyte inoculation and root biomass, with one study showing an endophyte-associated reduction in root weight in drought stressed barley (Murphy *et al.* 2015a) and another showing an endophyte-associated increase in root weight in optimally grown plants (Kumar *et al.* 2012). Murphy *et al.* (2015) report a neutral response in root weight due to endophyte inoculation in nutrient-stressed plants. Taken together, these contrasting results indicate that simultaneous multiple stresses induce a different response in root tissue allocation than a single stress. Since field conditions present multiple rather than single stresses for crop plants, our results may more accurately reflect plant responses to endophyte inoculation in agricultural situations (particularly as we also used a soil-based growing compost).

While there was no obvious endophyte effect on the degree of *Gaeumannomyces graminis* var. *tritic* infection in the post-harvest tissue, this may partly be due to the growing conditions used in this study. In general, this particular cool-temperate strain of *Gaeumannomyces graminis* var. *tritici* prefers much lower temperatures, higher moisture and a longer time to fully develop pathogenicity (Bockus & Tisser at 2000; Mathre 2000; Cook 2003; Mehta 2014). The high temperature and extremely low moisture in our

experiment may have either completely halted development and spread or even killed it off altogether. The barley cultivar that we used, Propino, does have good resistance to foliar disease, and an endophyte effect on this pathogen in the early stages of growth cannot be ruled out.

Plant responses to different stresses are highly complex, and recent evidence shows that plants respond to multiple stresses differently from how they do to individual stresses (Atkinson & Urwin 2012), with the interaction between biotic and abiotic stresses orchestrated by plant hormones and regulatory networks of molecular mechanisms such as transcription factors and reactive oxygen species (Langridge *et al.* 2006). The 'additional interactions' associated with endophyte infection serves to increase this complexity even further. The specific mechanisms associated with the improvements in agronomic traits that we and others have found are presently not known (Singh *et al.* 2011). While there have been studies examining changes in plant responses induced by the model fungal root endophyte *Piriformospora indica* and others (Schulz *et al.* 1999; Waller *et al.* 2008; Molitor & Kogel 2009; Lahrmann & Zuccaro 2012), further work using gene expression arrays and hormone cross-talk may elucidate the particular mechanisms associated with the stress related symbiosis that we have studied. It must also be noted that plant responses such as photosynthetic performance are cultivar-related (Afshari-Behbahanizadeh *et al.* 2014).The endophytes were derived from plants growing in very dry and nutrient poor soils (Murphy *et al.* 2014a) similar in several respects to the experimental conditions, so there may be a habitat-related selection of beneficial endophytes for these particular conditions(Rodriguez *et al.* 2008).

The positive benefits for barley related to endophyte inoculation that we have found has real significance for barley growers, particularly in the light of probable future changes in regional climate associated with global change. Where crop performance may currently only be limited by a single stressor, the addition of another climate change related stress may make the cultivation of the crop unviable. It is also desirable to reduce crop fertiliser inputs, both to save money and relieve environmental damage, but this will be difficult to achieve due to increasing demand for food. Endophyte treatments may provide part of the solution. We have shown for the first time that novel endophytes can even make the difference between life and death for multiply-stressed barley.

V. Conclusions

While controlled environment experiments using single plant stress factors have limited predictive value when applied to complex field conditions, multiple stress experiments with multiple endophyte inoculants more closely reflect the conditions that the cereal crop encounters in real agricultural environments. We have demonstrated that fungal root endophytes, derived from congeneric wild relatives of barley, have real potential in alleviating these stresses and could become particularly important for survival under future climate change scenarios. Future research should focus on translating results from controlled environment experiments using single endophyte inoculants of barley and other crops under single stresses to developing field crop inoculants using multiple endophytes.

Note

A patent for the use of these endophytes has been filed by Trinity College Dublin, and the use of these endophytes for biofertilisation and biocontrol purposes in cereal crop plants by third parties is subject to negotiated agreement with Trinity College Dublin and they may not be used without such permission.

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Figure captions

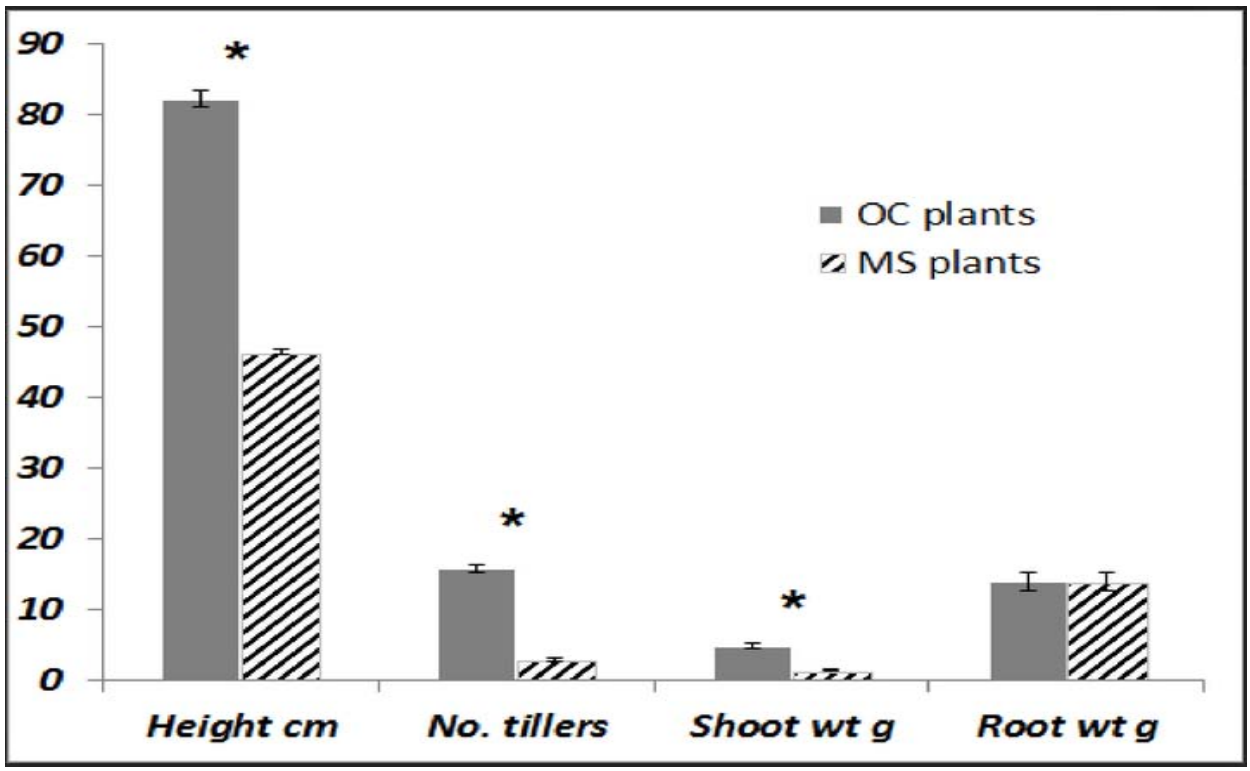

Figure 1 : Mean harvest values for selected barley traits between barley grown under optimal conditions (OC) and under multiple stresses (MS). Items marked with '*' indicate significantly greater values for OC treatment ($P < 0.01$) (n=15)

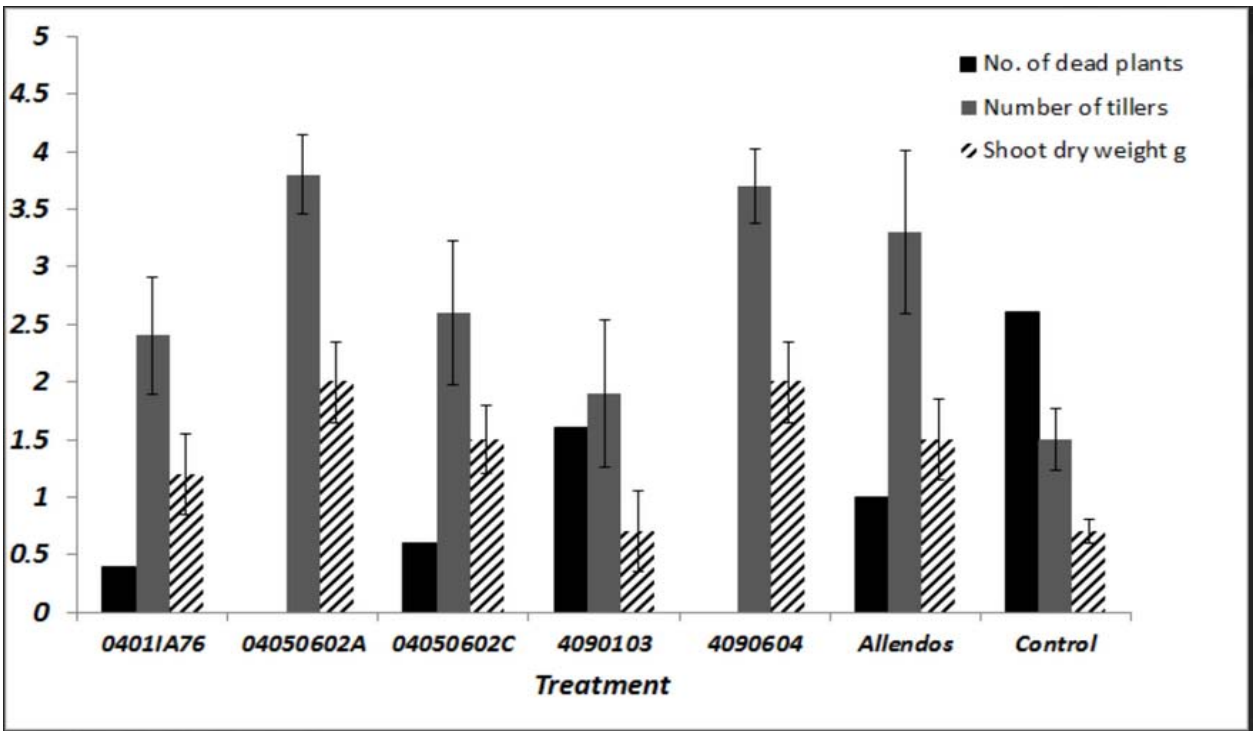

Figure 2 : Number of dead plants per pot of 3 plants, number of tillers per plant ± S.E. and shoot dry weight per plant ± S.E. for barley grown under multiple stresses (MS) (n=15)

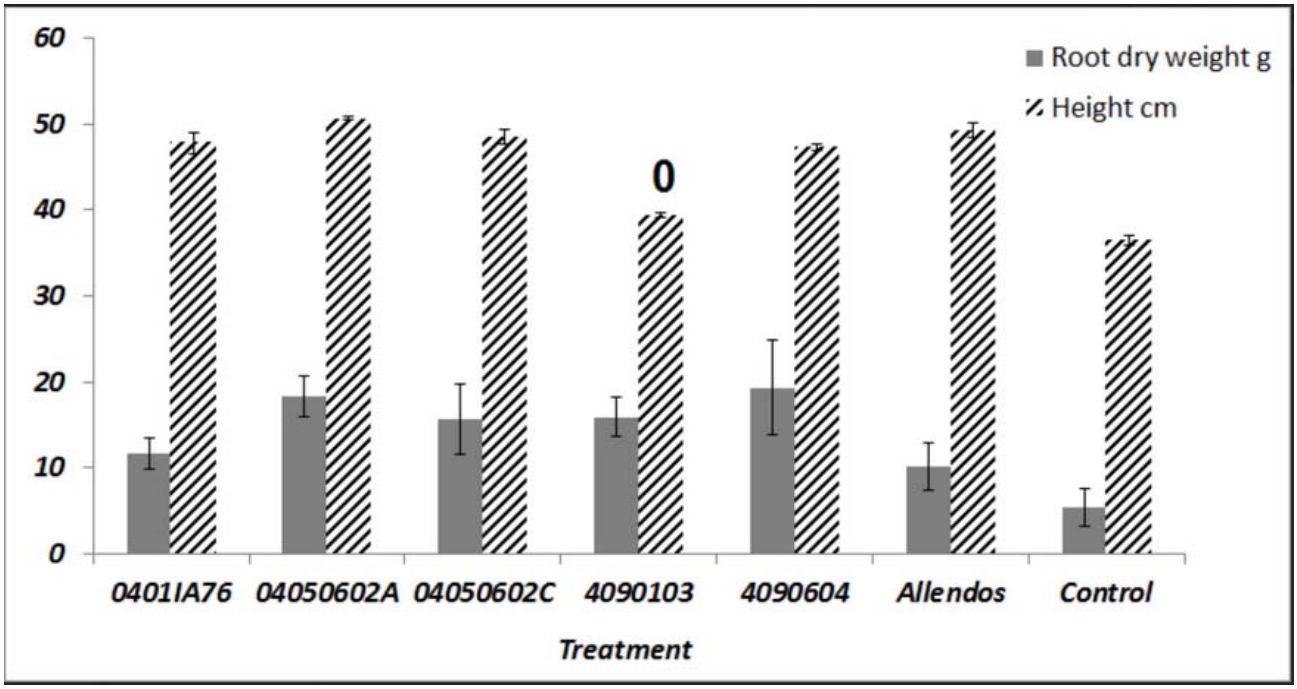

Figure 3 : Mean root dry weight ± S.E.and mean plant height ± S.E.for barley grown under multiple stresses (MS). All values for endophyte treatments, except those marked with '**0**', are significantly greater ($P < 0.05$) than the control (n=15)

Table 1 : ITS sequence relationship of endophyte strains with known GenBank accessions.

Endophyte strain	Gen Bank Accession	Nearest BLAST Match	% pairwise similarity
040605(2)A	KM492844	Uncultured *Cladosporium* clone, JF449686	82
040605(2)C	KM492845	*Penicilliumglabrum*, JN887323	85
0401IA76	KM492846	*Lophiostomacorticola*,HM116751	85
040901(3)	KM492837	*Penicilliumbrevicompactum* EU587331 [1]	95
040906(4)	KM492839	Uncultured *Metarhizium* KC797571 [2]	95

Table 2 : Significant negative (--) and positive (++) differences in barley traits between endophyte-inoculated and control plants grown in optimal conditions (OC) and under multiple stress (MS); 0 indicates no difference, * indicates $p < 0.05$, ** indicates $P < 0.01$ (n=15).

Trait	OC/MS	Endophyte difference, less (--) or greater (++) than control						Overall
		0401IA76	*040605(2)A*	*040605(2)C*	*040901(3)*	*040906(4)*	*Allendos*	
Germination Index	OC	0	0	0	++, *	0	0	0
	MS	0	++, **	++, **	0	0	0	0
Mean number of dead plants per pot	OC	0	0	0	0	0	0	0
	MS	++, **	++, **	++, **	++, **	++, **	++, **	++, **
Mean height	OC	0	++, **	++, **	++, **	++, **	++, **	++, **
	MS	++, **	++, **	++, **	++, **	++, *	++, **	++, **

Mean shoot dry weight	OC	++, **	0	0	--, **	0	0	0
	MS	++, *	++, **	++, **	0	++, **	++, **	++, *
Mean root dry weight	OC	--,*	--,*	--,*	--,*	0	--, *	--,*
	MS	++,**	++,**	++,**	++,**	++,**	++, **	++,**
Mean number of tillers	OC	++, *	++, *	0	++, *	0	++, *	++, *
	MS	++, *	++, *	++, **	0	++, **	++, **	++, *
Overall difference	OC	++, *	++, *	0	0	0	0	0
	MS	++,*	++,**	++,**	++,*	++,**	++, **	++,**

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Bacteria Associated with Pneumonia in Camels (*Camelus Dromedarius*) in the Sudan and Sensitivity of Some Isolates to Antibiotics using Vitek 2 Compact

By Muna E. Ahmed, Musa Tibin Musa & Mohammed A. E

Abstract- *Aims:* This study is to isolate and characterized the bacterial isolates associated with pneumonia in Sudanese camels using different methods and test the sensitivity of some isolates to antibiotics.

Study design: A total of 800 affected lung samples were collected from 400 camels (Camelus dromedarius) of different ages, seasons and part of the Sudan.

Place and Duration of Study: The study was undertaken in the laboratories of the Veterinary Research Institute, Ministry of animal resources and fisheries during 2009 to 2013.

Methodology: The bacterial isolates were characterized by three different methods: the conventional, Api kits and automated Vitek 2 Compact System. Fourteen of the Gram negative and 11 of the Gram positive bacterial isolates were tested for sensitivity to antibiotics by the Vitek 2 Compact using a sensitivity card which contained 20-21 antibiotics

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Results: A total of 713 bacterial isolates were isolated from the specimens of which 489 (68.6%) were Gram positive and 224 (31.4 %) were Gram negative. that were carried on 584 (81.90%), 60(8.42%) and 69 (9.68%) of the isolates, respectively. Antimicrobial resistance and sensitivity test were discussed; Ciprofloxacin is a drug of choice for *Staphylococcus* and enterobacteria spp treatments, but recently some bacterial species developed resistance to this antibiotic. Although cefazolin is an important antibiotic for human treatments and is given in severe cases, resistance of different bacteria spp to this antibiotic is regarded as a serious problem.

Conclusion: *Staphylococcus* spp in this study were isolated from all types of pathological lesions described and they were the most prevalent organisms (30.4%). Antibiotics used for treatments of pneumonic cases in camels should be effective against the causative bacteria and the public health aspects must be considered.

I. Introduction

Population of camels in the Sudan ranks the second in the world after Somalia and it is about 4.5 millions head. Probably the camel entered the Sudan through the following routes: North West Africa during the forth to the sixth century, Egyptian and the Red Sea routes[1].The camels are distributed in the arid and semi-arid areas north of latitude 13°N in the country [2]. Camels are owned by nomadic tribes like Arigat, Bani Amer, Bataheen, Bija, Hadandawi, Hamar, Hawaweer, Kababishi, Kawahla, Kenana, Lahaween, Magarba, Rashaida, Rizigat, Shukria and Zagawa, who took complete responsibility and care of their animals [3]. Viruses, bacteria, fungi and parasites were incriminated as the main causative agents of pneumonia in mammals; these agents may represent risks to camels, other livestock and even human populations [4]. The microorganisms could also be found as commensally in the upper respiratory tracts of animals and these could play a pathogenic role when general resistance of the host is lowered.

Rearing systems, stress factors, climate changes, and unhygienic conditions, sudden changes in feed and low level herd health status were stated to be risk factors associated with bacterial and viral pneumonia. In camels, pneumonia outbreaks were usually observed during the change from dry to rainy seasons [5].

Aims of the study

* Isolate bacteria associated with pneumonia and the upper respiratory tract infections in camels slaughtered at Tambool slaughterhouse.
* Characterize the bacterial isolates using the conventional bacteriological methods, API kits and vitek 2 compact system (Biomerieux, France).
* Test sensitivity of the some isolates to antibiotics with the Vitek 2 Compact instrument.

II. Materials and Methods

a) Collection of samples

Eight hundred samples were collected from 400 camels, of ages ranging from 6 months to 15 years and originated from different parts of the Sudan including Kassala, AlGadarif, Kordofan and Darfur states and slaughtered at Tambool abattoir. From each camel an affected lung specimen and tracheal swab were collected for bacterial isolation and identification. The samples were collected aseptically, each in a sterile plastic bag and transported immediately on ice to the

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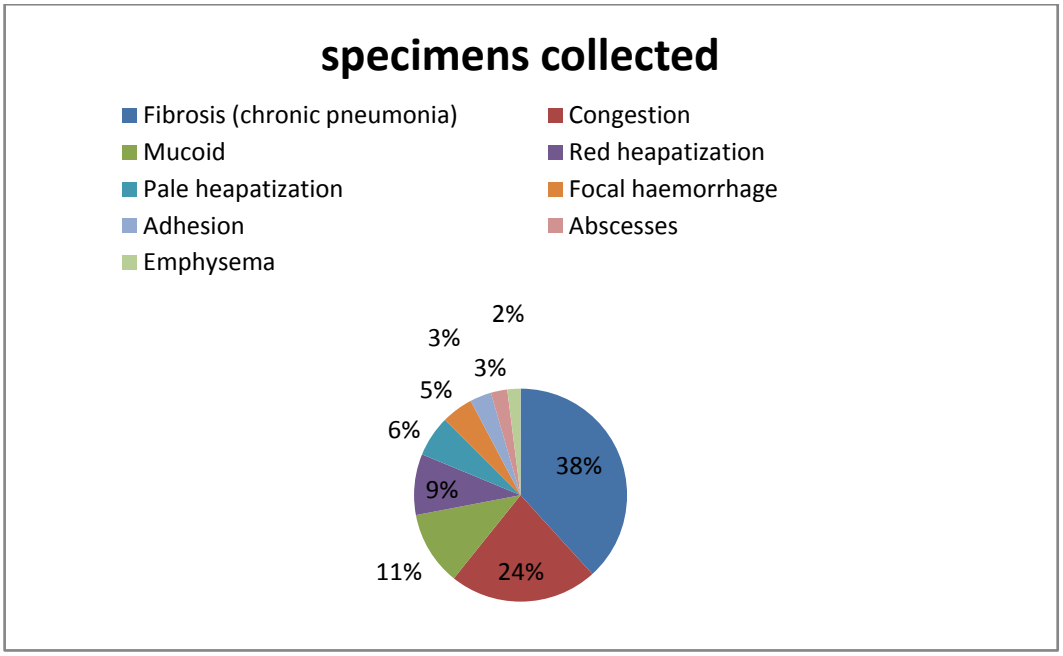
Veterinary Research Institute, Department of Bacteriology for isolation and identification of bacteria associated with the pneumonias (fig 2- 3).

Table (1) : Pathological lesions in lungs specimens collected for the study



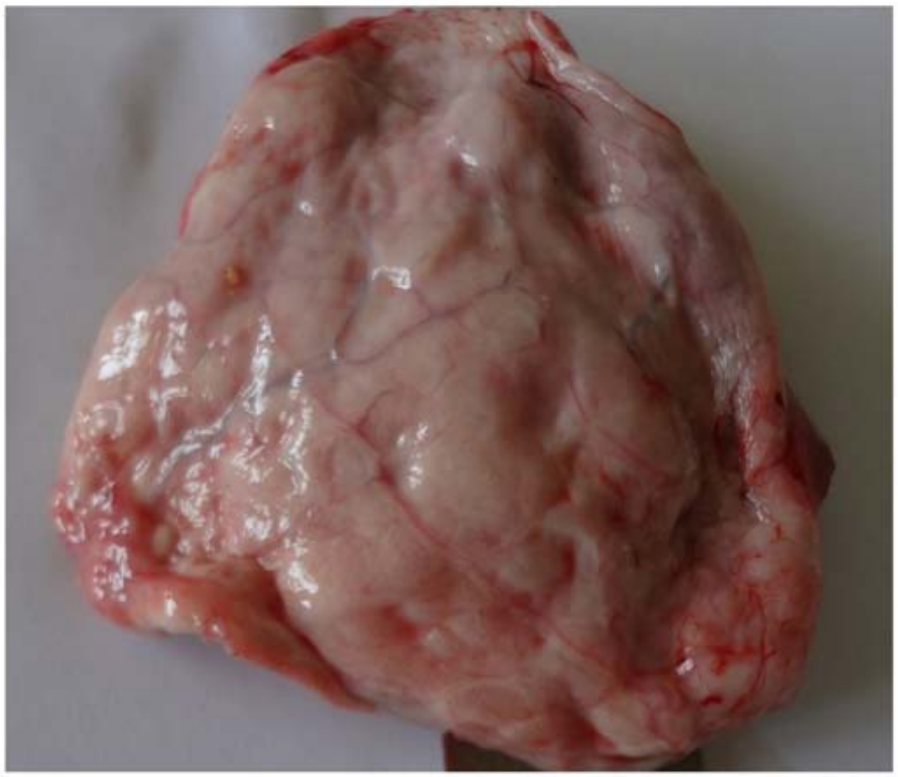


Figure 1 : A camel suppurative pneumonic lung from which *R. equi* and *Ps. aeruginosa* were isolated.

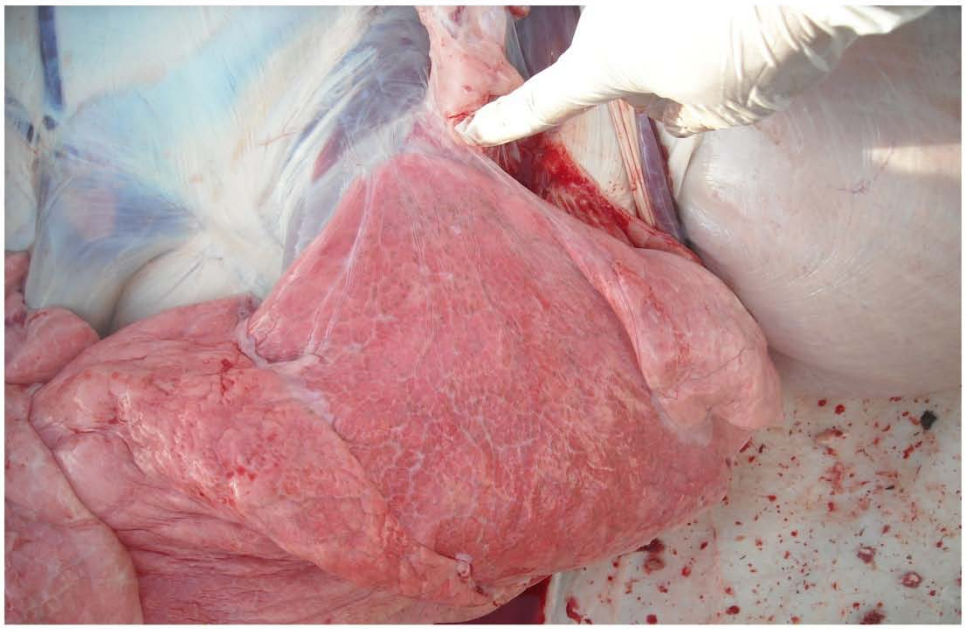


Figure 2: A camel lung specimen adhered to plerum from which *S. auerus*, *S.epidermidis* and *Str. pyogenes* were isolated.

b) Cultural procedure

Different types of media were used for isolation of the bacteria from the specimens collected. Those were: nutrient agar , blood agar, brain heart infusion, mannitol salt agar, MacConkey agar and brilliant green agar .The surface of each lung sample was cauterised with a red hot scalpel blade for decontamination. A deep incision was then made in each lung surface using sterile scalpel blade, a sterile swab was dipped into the incised area and streaked onto sheep blood agar (Oxoid CM271) plate. From the incised area a piece of sample was cut and put in brain heart infusion broth in a bijou bottle. The cultures were incubated aerobically at 37°C for 24hours. Any plate that did not show growth within 24hr was incubated for a week and examined daily to ensure bacterial growth before considering it negative. Each tracheal swab was placed into brain heart infusion broth medium and incubated at 37°C for 24 hr. The isolates recovered were identified at the generic and species levels. Different procedures were used for the identification of the isolates: the conventional method, Api kits method and Vitek 2 Compact automated system. A pure culture of each isolate was subcultured onto a blood agar slant and after incubation at 37°C for 24 hr, stored at 4°C till used for identification.

c) Conventional methods

i *Primary biochemical tests*

Smears were prepared from each fresh culture of the 800 samples grown aerobically and stained with the Gram stain for reaction, morphology, non acid fastness and spore formation. The cultures were also tested for motility using hanging drop and Craige tube methods. The other tests performed were catalase, oxidase, acid or acid and gas from glucose and oxidation fermentation test [6]. According to the results of the primary tests, secondary tests were determined and performed for each isolate to be identified to the species level [6].

ii *Secondary biochemical tests for the Gram positive*

The secondary tests for the Gram positive isolates were: catalase, oxidase and indole production, motility test, coagulase test, carbohydrates breakdown, Voges-Proskauer reaction, arginine hydrolysis, nitrate reduction , growth in 6.5% NaCl broth, growth at 45°C, requirement of CO_2 for growth, sensitivity to bacitracin (0.1 unit), urease activity, gelatin liquefaction and aesculin hydrolysis .

iii *Secondary tests for the Gram negative*

The secondary tests for Gram negative isolates were: oxidase production, citrate utilization, urease activity, growth in KCN medium, gelatin liquefaction and hydrogen sulphide production from the TSI medium, fermentation of sugars, growth at 42°C, growth on MacConkey agar, nitrate reduction, indole production, aesculin and arginine hydrolysis, Voges-Proskauer reaction and the methyl red test. Other tests were used for Gram positive and Gram negative whenever indicated by [6.7].

d) Characterisation of the Staphylococci isolates using API Staph

For identification of 25 *Staphylococcus* isolate with the Api kits, the organisms were subcultured on blood agar plates, each separately and after incubation

for 24hours at 37°C, colonies from each fresh culture, were emulsified in Api Staph medium and adjusted visually to 0.5 MacFarland opacity tube that was prepared by adding 0.05 of 1.0% barium chloride to 9.95 of 1.0% sulfuric acid.

e) Identification by the Vitek 2 Compact

The card for each Gram positive or negative group of bacteria was automatically filled by a vacuum device, sealed and inserted into the Vitek 2 reader – incubator module (incubation temperature 35.5°c) and subjected to a kinetic colorimetric measurement every 15 min. Data were analyzed using Vitek 2 database version 4.01. All cards used were automatically discarded into a waste container. Final identification results were available in approximately 8 hours or less in Gram positive bacteria or 10 hours or less in Gram negative bacteria.

i. *Analysis of the Vitek 2 Compact result*

There were four possibilities for analysis of identification results: (1) correct identification in which the strains were correctly identified to the species level or strains with low discrimination were resolved. (2) Low discrimination, in which the strains with low discrimination were not resolved by simple additional tests. (3) Misidentification, in which discrepant results obtained for the strains were different from those identified by the reference method and (4) No identification was provided. The mean time for the results generation was also calculated for all identifications.

f) Antibiotic Sensitivity tests (AST) with the Vitek 2 Compact

The 0.5 McFarland bacterial suspension was diluted by adding 280µl of the suspension to 3ml 0.45% saline in the AST for Gram positive organisms and 145µl of the suspension to 3 ml in the AST for Gram negative organisms. The cards were automatically filled, sealed and loaded into the Vitek 2 Compact for incubation and reading. The AST-P586-card for *Staphylococcus* and *enterococcus* contained: benzylpenicillin, ampicillin, oxacillin, impenem, cefoxitin, cefoxitin screen, gentamicin high level, streptomycin high level, gentamicin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin, inducible clindamycin resistance, linezlid, teicoplanin, vancomycin, tetracycline, tigecycline, fosfomycin, fusidic acid, rifampicin, and trimethoprim/ sulfameythoxazole. The card for the gram-ve isolates AST-GN30- contained ampicillin, ampicillin/sulbactam, piperacillin/tazobacam, cefazolin, cefoxitin, ceftazidime, ceftriaxone, cefepime, imipenem, metropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin, and trimethoprim/ sulfamethoxazole.

i. *Analysis of susceptibility tests using the Vitek 2 Compact system*

There were two possibilities for analysis of susceptibility testing: (1) Category agreement (CA), In CA the microbial susceptibility was determined as susceptible, intermediate, or resistant according to National Committee of Clinical Laboratory Standards (CLSI), (2) Discrepancies which considered major errors when the system indicated intermediate susceptibility and the reference method indicated susceptibility or resistance or when the system indicated susceptibility or resistance and the reference method indicated intermediate susceptibility [8].

III. Results

From the 800 samples, 713 bacterial isolates were obtained from the camel tracheal swabs and pneumonic lungs. The highest incidences of pneumonic cases were found in autumn and were 247(61.75%) and the lowest ones in Summer and were 153(38.25%). The adult camels were more susceptible 307(76.75%) than the younger ones 93(23.25%). The types of bacteria isolated from the different types of lesions are presented in Table 1. A total of 584(81.77%), 69(9.68%) and 60(8.42%) isolates were identified by the Conventional, Vitek 2 Compact and Api kits, respectively (Table 2).

Table 2 : Bacteria isolated from the tracheal swabs and lung lesions in the camels examined

Lesion of the lungs/ site	Bacteria recovered
Congestion	*Staphylococcus* spp, *Corynebacterium* spp, *Pseudomonas* spp, *K.pneumoniae* and *E.coli*
Hepatization	*Staphylococcus* spp, *Streptococcus* spp, *K.pneumoniae, E.coli* and *Corynebacrerium* spp.
Abcesses	*Staphylococcus* spp, *Streptococcus* spp, *Actinomyces* spp, *Micrococcus* spp and *Ps.aeruginosa*
Supprative (muocoid)	*R.equi, Corynebacterium* spp, *Ps.aeruginosa, K.pneumoniae, Burkholderia* spp, *S.agalactiae* and *S.epidermidis*
Adhesion	*Aeromonas spp, Streptococcus* spp and *Staphylococcus* spp
Tracheal swabs	*Staphylococcus* spp, *Streptococcus* spp, *Micrococcus* spp, *E.coli*, *Corynebacterium* spp, *Alloicoccus* sp and *B.bronchiseptica*

Table 3 : Bacterial isolates identified by the different methods

Isolate	Conventional method	Api kits	Vitek 2 Compact	Total No.
Staphylococcus spp	174(24.40%)	25(3.51%)	17(2.38%)	216 (30.40%)
Streptococcus spp	94(13.18%)	20(2.81%)	11(1.54%)	125 (17.60%)
Enterobacteria spp	148(20.76%)	10(1.40%)	12(1.68%)	170 (23.90%)
Micrococcus spp	49(6.87%)	_	4(0.56%)	53 (7.50%)
Corynebacteria spp	27(3.79%)	_	2(0.30%)	29 (4.00%)
Pseudomonas spp	17(2.39%)	1(0.14%)	3(0.42%)	21 (3.00%)
Actinomyces spp	15(2.10%)	_	2(0.30%)	17 (2.40%)
Aeromonas spp	14(1.97%)	2(0.28%)	6(0.84%)	22 (3.10%)
Alloiococcus otitis	_	_	2(0.30%)	2 (0.30%)
Facklamia hominis	_	_	1(0.10%)	1 (0.10%)
Bordetella bronchiseptica	_	_	1(0.10%)	1 (0.10%)
Leuconostoc pseudomesenteroides	_	_	1(0.10%)	1 (0.10%)
Pediococcus sp	_	_	1(0.10%)	1 (0.10%)
Stenotrophomonas maltophia	_	_	1(0.10%)	1 (0.10%)
Sphingomonas paucimobilis	_	_	3(0.42%)	3 (0.42%)
Burkholderia cepacia	_	2(0.28%)	2(0.30%)	4 (0.60%)
Bacillus spp	45(6.31%)	_	_	45 (6.30%)
Gardnerella vaginalis	_	_	1(0.10%)	1 (0.10%)
Total No. of bacteria	584(81.91)	60(8.42%)	69(9.67%)	713 (100%)

Table 4 : Identification of the *Staphylococcus* species by the Conventional, Vitek 2 Compact and Api kits

No. of isolates characterized	Conventional test	Vitek 2 procedures	Api Staph kits
S.aureus	43 (24.71%)	5 (29.42%)	10 (40%)
S.epidermidis	35 (20.11%)	2 (11.77%)	6 (24%)
S.intermedius	18 (10.34%)	2 (11.77%)	1 (4%)
S.hyicus	10 (5.75%)	1 (5.88%)	2 (8%)
S.chromogenes	6 (3.45%)	1 (5.88%)	_ (0.0%)
S.warneri	25 (14.37%)	1 (5.88%)	2 (8%)
S.saprophyticus	9 (5.17%)	1 (5.88%)	1 (4%)
S.haemolyticus	2 (1.15%)	1 (5.88%)	_ (0.0%)
S.lentus	12 (6.90%)	1 (5.88%)	1 (4%)
S.simulans	13 (7.47%)	1 (5.88%)	2 (8%)
S.hominis	1 (0.58%)	1 (5.88%)	0 (0.0%)
Total	174 (100%)	17 (100%)	25 (100%)

Table 5 : Identification of the *Streptococcus* species by the Conventional, Vitek 2 Compact and Api kits

Isolates	Conventional tests	Vitek 2 Compact procedures	Api20S kit
Str.pyogenes	14 (14.89%)	_ (0.0%)	_ (0.0%)
Str.pneumoniae	11 (11.70%)	_ (0.0%)	5 (25%)
Str.agalactiae	_ (0.0%)	2 (18.18%)	3 (15%)
Str.bovis	4 (4.26%)	2 (18.18%)	3 (15%)
Str.uberis	7 (7.45%)	_ (0.0%)	2 (10%)
Str.sanguis	9 (9.57%)	2 (18.18%)	3 (15%)
Str.suis	_ (0.0%)	1 (9.09%)	_ (0.0%)
Str.alactolyticus	_ (0.0%)	1 (9.09)	_ (0.0%)
Str. viridans	20 (21.28%)	_ (0.0%)	_ (0.0%)
E.faecium	16 (17.02%)	2 (18.18%)	2 (10%)
E.faecalis	13 (13.83%)	1 (9.09%)	2 (10%)
Total	94 (100%)	11 (100%)	20 (100%)

Other isolates were: *Escherichia coli* :one hundred twenty nine isolates were identified conventionally, two by Vitek 2(0.28) Compact and 4 (0.56%) by Api 20 E kits , *Klebsiella pneumoniae*: Ninteen (2.67%) of *K.pneumoniae* strain were identified conventionally, 3 (0.42%) with the Vitek 2 compact and 3 (0.42%) with the Api kits, *Pseudomonas aeruginosa*: 17(2.39%) of the *Ps. aeruginosa* were isolated conventionally, 3(0.42%) with Vitek 2 Compact and one (0.14%) with the Api kit, *Sphingomonas paucimobilis*: Three strains (0.40%) of this organism were identified using the Vitek 2 Compact, *Bordetella bronchiseptica*: One (0.10%) strain of this organism was identified by theVitek 2 Compact *Aeromonas salmonicida* and *Aeromonas hydrophilia*: 14 (1.97%) *Aeromonas* spp were identified conventionally, 6(0.84%) with the Vitek 2 Compact and 2 (0.28%) with the Api 20 E kits. The other Gram negative rods were 4(0.56%) *Enterobacter cloacae* (*E. cloacae*), 2 (0.28%) of each *E. sakazakii and Acinetobacter* spp, 1 (0.14%) each of *Morganella morganii*, *Pantoe* sp, *E. herrmannii*, *Stenotrophomonas maltophilia* and *Providencia stuartii*.

Ten isolates were characterized with the three methods: Conventional, Api kits and Vitek 2 Compact for comparison, they were *S. aureus*, *S. epidermidis, S. hycus*, *S. warneri*, *Str. bovis*, *E. faecalis*, *Ps. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. salmonicida* strain. The same results were obtained but in it took different time.

a) *Antibiotics sensitivity tests (AST) using Vitek 2 Compact*

Twenty five isolates were used for AST with the Vitek 2 Compact; 14(1.96%) of the organisms were Gram negative and 11(1.54%) were Gram positive. The Gram negative were 2(0.28%) each of *K. pneumoniae*, *Ps. Aeruginosa*, *Acinetobacter* spp and *B. cepacia* and 1(0.14%) each of *E. cloacae*, *B. bronchiseptica* and *A. hydropila* and 3(0.42%) *E. coli*. The Gram positive were 3(0.42%) *S. aureus* and 1(0.14%) each of *S. epidermidis*, *S. intermedius*, *S. hyicus*, *S. haemolyticus*, *S. chromogenes*, *E. faecalis* and *E. faecium*.

Table 6 : Antibiotics sensitivity tests to the Gram positive bacteria

Antibiotics	1	2	2	3	4	5	6	7	8	9
Benzylpenicillin		R		R	S	R	S	R	R	
Ampicillin	R								R	S
Oxacillin		R		S	S		S	R		
Cefoxitin Screen		+			_	_		_		
Gentamicin high level									S	S
Streptomycin high level									S	S
Gentamicin		S		S	S	S	S	S		

Ciprofloxacin		S		S	S	S	S	S	S	S
Levofloxacin	I	S						S	S	
Moxifloxacin	S	S		S	S	S	S	S	S	S
Erythromycin	R	R		S	S	R	S	S	R	S
Clindamycin	R	R		S	S	R	S	S	S	R
Quinupristin/Dalfopristin	S	S						S	S	
Inducible Clindamycin Resistance		+		–	–	–	–			
Linezolid	S	S		S	S		S	S	S	S
Vancomycin	S	S		S	S	R	S	S	S	S
Tetracycline	R	R	R	R	S	S	S	S	R	S
Tigecycline	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin	S	S	S					S	S	
Rifampicin		S						S		
Trimethoprim/Sulfamethoxazole	R	S	S	S	S	S	S	S		R

Key:

1: *S.haemolyticus* 2: *S.aureus* 3: *S.epidermidis* 4: *S.intermedius* .

5: *S.simulans* 6: *S.hyicus* 7: *S. chromogenes* 8: *E.faecalis* 9: *E. faecium* S: Sensitive R: Resistant

: not tested

Table 7 : Antibiotics sensitivity tests to the Gram negative bacteria

Antibiotics	K 1	K 2	B 1	B2	E1	E2	P 1	P 2
Ampicillin	R	_	S	R	R	R	R	R
Ampicillin/Sulbactam	S	S	S	R	R	_	R	R
Piperacillin/Tazobactam	S	S	S	S	R	I	S	S
Cefazolin	S	S	R	R	R	_	R	R
Cefoxitin	S	S	R	R	R	S	R	R
Cefazidime	S	S	S	S	R	S	S	S
Ceftriaxone	S	S	S	R	R	_	R	R
Cefepime	S	S	S	I	R	R	S	S
Imipenem	S	S	S	R	R	S	S	S
Meropenem	S	S	S	I	R	_	S	S
Amikacin	S	S	R	R	R	S	S	S
Gentamicin	S	S	S	R	R	R	S	S
Tobramycin	S	S	S	R	R	R	S	S
Ciprofloxacin	S	S	S	I	R	R	S	S
Levofloxacin	S	S	S	I	R	_	S	S
Nitrofurantoin	S	S	R	R	R	S	R	R
Trimethoprim/Sulfamethoxazole	S	S	S	S	R	S	R	R

Key:

K I, K 2: K.pneumoniae *B 1, B 2: B.cepacia*

E 1, E 2: E.coli *P 1, P 2: Ps.aeruginosa*

S: Sensitive *R: Resistant* *I: Intermediate - : Not tested*

Table 8 : Antibiotics sensitivity tests to the Gram negative bacteria

Antibiotics	A.hydr	E.cloa	B.bro	Acit 1	Acit 2
Ampicillin	R	S	I	R	I
Ampicillin/Sulbactam	S	S	I	I	I
Piperacillin/Tazobactam	S	S	S	S	S
Cefazolin	R	R	R	R	R
Cefoxitin	R	R	R	R	R
Cefazidime	S	R	S	S	S
Ceftriaxone	I	S	S	S	S
Cefepime	I	S	S	S	S
Imipenem	S	S	S	S	S
Meropenem	S	S	S	S	S
Amikacin	R	R	S	S	S
Gentamicin	R	R	S	S	S
Tobramycin	R	R	S	S	S
Ciprofloxacin	I	S	S	S	S
Levofloxacin	I	S	D	S	S
Nitrofurantoin	R	I	R	I	R
Trimethoprim/Sulfamethoxazole	S	R	S	S	S

Key:

A.hydr : A.hydrophila *E.cloa: E.cloaca*

B.bro: B.bronchseptica *Acit: Acitinobacter spp*

S: Sensitive R: Resistant I: Intermediate

IV. Discussion

Staphylococcus spp in this study were isolated from all types of pathological lesions described and they were the most prevalent organisms (30.4%). In a similar study in camels, [9] found 16.6% of his isolates to be *Staphylococcus* spp and [10] found 27.9% of this organism. *The Streptococcus* spp represented 17.6% of the isolates, while [5] found them to be 5.33%, [11], 7% and [12] 19.3% of their isolates from camels.

In this paper *E. coli* were 18.9% of the isolates. This organism was also isolated by [12, 5] who found 17.5% and 26.7% of their isolates from camels were *E.coli*. Isolation of *E.coli* in these rates indicates the importance of the organism in respiratory tract infections of camels. Although *Micrococcus* spp are regarded as non-pathogic bacteria *M.luteus* had been implicated as the causative agent in cases of meningitis, abscesses, pneumonia and septic arthritis in immunosupresed patients [13]. In this study, *Kocuria rosa* (formally *M.rosa*) which was isolated from lung abscesses (Table1), was recently reported as one of catheter related bacteria. It was isolated in a pure culture from patient with stem cell transplantation and this uncommon pathogen may cause opportunistic infections in immunocompromised patients [13].

The *A. pyogenes* was also isolated from camels by [9]. The organism was not previously reported in camels as a respiratory tract pathogen. It caused suppurative lesions and abscesses in various organs and tissues mainly lungs [14]. It was also isolated from lungs of ruminants, pigs and sometimes people [15].

The *B. bronchiseptica* which was isolated from a trachea swab of a camel (Table1) was also isolated previously by [16] from tracheas and lungs of camels. The isolation of *R. equi* from a camel in this study is in agreement with [16,12] who isolated the organism from camels. The organism usually causes bronchopneumonia in foals. The *Bacillus* spp isolated were 6.3% of the total isolates. [9, 10] found 5% and 12.8%, respectively of the organisms among the different isolates from camel examined.. The *Leuconostoc pseudomesenteroides* is an uncommon pathogen of the respiratory tracts of camels. It was also isolated by [17] from patients with pneumonia. The *Ps, aeruginosa* represented 3% of the total isolates from the camels. This reflects a public health importance that camel could pertain. [18] found 1.07%, 5% and 12% respectively of the organisms isolated from camel to be *Ps, aeruginosa*. The *S. paucimbilis* which was isolated from the pneumonic lungs of the camels, was also

isolated by [19]. Isolation and identification of *Str. agalaciae*, *Str. suis*, *Str. bovis* and *A.otitis* were dealt with in previous paper [20].

Conventional methods were considered as a baseline testing system for isolation, whereas Api kits and automated Vitek 2 compact were better off in terms of identification and characterization of bacteria. In the present study, three methods of identification were used: The conventional, by which 584 (81.91%) isolates were identified, Api kits 60 (8.42%) and Vitek 2 Compact 69 (9.67%). The conventional biochemical tests for identification of bacteria are cumbersome, use limited biochemical tests, liable to contamination, take a long time and can result in inconclusive results [6]. The Api diagnostic kits are considered a gold standard tests for identification of bacteria [21]. The kits consist of 20 micro tubes which contained dehydrated substrates. They are easy to use, have extensive data base of characteristics and standardized biochemical reactions of microorganisms and the results are ready after 24hr. Vitek 2 Compact system which is a fully automated system is more developed because it contains 64 biochemical tests to identify organisms and grades the type of identifications whether they are excellent, good or acceptable and gives details of biochemical tests. The system is better than the Api kits in terms of accuracy for identifications. Also it has analysis expert system (AES) which analyzes the antibiotic sensitivity test results using the well established knowledge based on approximately 100 species and 20000 ranges of minimum inhibitory cells to detect more than 2300 phenotypic antimicrobial resistance. The Vitek 2 Compact and AES were evaluated in several countries [22]. The Gram positive organisms which tested in this study against antibiotics were sensitive to gentamycin, moxifloxacin, linezolid, tigecycline and ciprofloxacin. Of the Gram negative strains, a strain of *E. coli* was found to be resistant to all antibiotics. *Ps. Aeruginosa* was resistant to ampicillin, cefazolin, cefoxitin, ceftraxone, nitrofurantoin and trimethoprim/sulfamethazole; *B. cepacia* was resistant to cefazolin, cefoxitin, ceftriaxone, imipenem, amikacin, gentamycin, tobramycin and nitrofurantoin; *Aeromonas hydrophilia*, *Enterobacter cloaca*, *Bordotella bronchiseptica* and Acinitobacter spp were resistant to cefazolin and cefoxitin. The cefazolin is an important antibiotic for human treatments and is given in severe cases; resistance of different bacteria to this antibiotic is a problem. Ciprofloxacin is a drug of choice for Staphylococcus and Enterobacteria spp treatments but recently some bacterial species developed resistance to this antibiotic.

V. Conclusion

Identification should be carried with either Vitek 2 Compact or Api kits for more accurate and reliable results, to save time for identification and overcome the problems of contamination and scarcity of the substrates used in the conventional tests. *Staphylococcus* spp in this study were isolated from all types of pathological lesions described and they were the most prevalent organisms (30.4%). Vitek 2 Compact system enables to isolate bacteria of public health importance. Camels were found to be reservoirs of eight bacterial species pathogenic to man. A strain of *E. coli* was found to be resistant to all antibiotics tested in this study. Antibiotic sensitivity tests resulted in resistance of bacteria of public health importance. Antibiotics used for treatments of pneumonic cases should be tested for sensitivity against the causative bacteria. Ciprofloxacin is a drug of choice for Staphylococcus and Enterobacteria spp treatments but recently some bacterial species developed resistance to this antibiotic. In selection of antibiotics for treatment of infected camels the public health aspect must be considered.

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A Predictive Fuzzy Expert System for Diagnosis of Cassava Plant Diseases

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Abstract- Cassava is an important tropical root cropwidely grown in many part of the world in a range of agro-ecological environments. The crop can be used for food and non-foods products. Cassava is capable of providing starch for use in drug industries, it is a stable source of dietary energy for more than 500 million. Nonetheless, despite the nutritional and economic significance of the cassava crop, the diseases incidence on cassava plantations is fast becoming a constraint in farmers' quest for a bountiful harvest. The efforts of agricultural extension agents seem not to be sufficient in tackling this menace since there is always a limit to how far the human capacity can be stretched in the face of highly demanding situations. Hence, this paper proposed the development of fuzzy expert system for predicting cassava plant disease. The system was developed with the help of fuzzy tool in MATLAB vs. 9. It employed 18 rules for the Cassava Mosaic, 27 rules for the cassava brown streak and 27 rules for cassava bacteria blightfor the classification and prediction of cassava plant diseases. This would provide immediate and instant information to the possible disease.

Keywords : *cassava diseases, symptoms, prediction, fuzzy expert system, fuzzy inference system.*

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A Predictive Fuzzy Expert System for Diagnosis of Cassava Plant Diseases

Awoyelu, I. O. [α] & Adebisi, R. O. [σ]

Abstract- Cassava is an important tropical root cropwidely grown in many part of the world in a range of agro-ecological environments. The crop can be used for food and non-foods products. Cassava is capable of providing starch for use in drug industries, it is a stable source of dietary energy for more than 500 million. Nonetheless, despite the nutritional and economic significance of the cassava crop, the diseases incidence on cassava plantations is fast becoming a constraint in farmers' quest for a bountiful harvest. The efforts of agricultural extension agents seem not to be sufficient in tackling this menace since there is always a limit to how far the human capacity can be stretched in the face of highly demanding situations. Hence, this paper proposed the development of fuzzy expert system for predicting cassava plant disease. The system was developed with the help of fuzzy tool in MATLAB vs. 9. It employed 18 rules for the Cassava Mosaic, 27 rules for the cassava brown streak and 27 rules for cassava bacteria blightfor the classification and prediction of cassava plant diseases. This would provide immediate and instant information to the possible disease. It would fast-track information service delivery on the part of the large-scale industrial cassava farmers which make use of it for emergency situations of disease outbreak on the farm, pending the arrival of the agricultural extension agent. The fuzzy expert system predicts accurately once the symptoms and conditions are quantized. The result showed that the system was able to effectively predict and classify cassava diseases considered.

Keywords: cassava diseases, symptoms, prediction, fuzzy expert system, fuzzy inference system.

I. Introduction

Cassava is the third largest source of carbohydrates for human consumption worldwide, providing more food calories per cultivated acre than any other staple crop. It is an extremely robust plant which tolerates drought and low quality soil. The foremost cause of yield loss for this crop is viral disease [1]. The plant grows in a bushy form, up to 2.4 meters high, with greenish-yellow flowers. The roots are up to 8 centimeters thick and 91 centimeters long. Two varieties of the cassava are of economic value: the bitter, or poisonous; and the sweet, or non-poisonous. Both varieties yield a wholesome food because the volatile poison can be destroyed by heat in the process of preparation. Cassava is the chief source of tapioca, and in South America a sauce and an intoxicating beverage are prepared from the juice. The root in powder form is used to prepare *farinha*, a meal used to make thin cakes sometimes called cassava bread. The starch of cassava yields a product called Brazilian arrowroot. In Florida, where sweet cassava is grown, the roots are eaten as food, fed to stock, or used in the manufacture of starch and glucose.

The economies of many developing countries are dominated by an agricultural sector in which small-scale and subsistence farmers are responsible for most production, utilizing relatively low levels of agricultural technology. As a result, disease among staple crops presents a serious risk, with the potential for devastating consequences. It is therefore critical to monitor the spread of crop disease, allowing targeted interventions and foreknowledge of famine risk.

An expert system is a system that could keep knowledge in its knowledge base as the system knowledge resources and manipulate that knowledge, so it could prepare the high level decision tool to the user that is called inference engine as the brain of the system. On the other hand, expert system could help people in many cases in order to get decision in solving a problem.

A number of cassava diseases is responsible for reducing the overall production of cassava to a great extent. A disease is an alteration of one or more ordered series of physiological processes as caused by irritation from some factors or agents resulting into loss of co-ordination in plants. An accurate diagnosis of cassava diseases is essential for the appropriate management of the plant. The diagnosis of cassava plant diseases requires highly trained and experienced experts but there is dearth of experts. Therefore, a system is needed to diagnose cassava diseases, predict their outbreak and prevent their spread. Hence this paper proposes an expert system for diagnosing and predicting cassava diseases which could also be used both by the farmer and the experts to train their students.

The rest of this paper is arranged as follows. Section 1 deals with the introduction, section 2 presents a review of the related works on the developments of expert systems in agricultural field. The methodology used to achieve the set objectives is described in section 3. Section 4 contains implementation of the proposed system and result discussion. Finally, section 5 presents the conclusion.

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II. Existing Works

Expert systems have been developed and applied in many fields like medicine, engineering, agriculture, physical sciences and business. In agriculture, expert systems are developed to diagnose the diseases and pests of various crops[2]. Farmers across the world face problems like soil erosion, increasing cost of chemical pesticides, weather damage recovery, the need to spray, mixing and application, yield losses and pest resistance. On the other hand, researchers in the field of agriculture are constantly working on new management strategies to promote farm success. In many countries today, farming has become technologically advanced and expert systems are widely used in the field of agriculture. In this way, farmers can get expert opinions on their specific problems like selection of most suitable crop variety, diagnosis or identification of livestock disorder, suggestion of tactical decisions throughout production cycle from the expert system [2].

There have been some existing works on the development of expert systems in the agricultural field. A myriad of expert systems for various crops such as wheat, rice, maize, sunflower, lime, tomato, apple, orange, soybean and cucumber have been developed [3].[4] developed AMRAPALIKA - an expert system for the diagnosis of pests, disease and disorders of Indian mango. The objective of this work is to provide computer-based support for agricultural specialists or planters. The expert system makes diagnosis on the basis of response/responses of the user made against queries related to particular disease symptoms. The knowledge base of the system contains knowledge about symptoms and remedies of fourteen (14) diseases of Indian mango tree appearing during fruiting season and non-fruiting season.

Web-based Fuzzy Expert System for Integrated Pest Management (IPM) in Soybean was proposed by [5]. The system was developed with an objective to provide IPM decision support to the planters through the Internet. Besides that, the system applied the application of the fuzzy logic in uncertainty management during pest identification as well for estimating pest activity level using a new adjustable operator introduced by the authors. Pest Control Expert System for Tomato (PCEST) was developed by [6]. The system involves two main subtasks, namely: diagnose and treat. The diagnose subtask finds out the causes of the growers' complaints, while the treat subtask finds out a treatment plan for these causes. [7] developed a web-based expert system for fish disease diagnosis called Fish-Expert. This web-based intelligent system can mimic fish disease expertise and diagnose a number of fish diseases with a user-friendly interface. The system has over 300 rules and 400 images and graphics for different types of diseases and symptoms. It can diagnose 126 types of diseases amongst nine species of primary freshwater fishes. The system is in pilot use by fish planters in the North China region.

Fuzzy logic has been used to solve numerous problems ranging from prediction rate to classification rate. In the modeling of crop disease and control, fuzzy logic was used to model and monitor crop diseases in developing countries of the world. Models of crop disease are used for understanding the spread or severity of an epidemic, predicting the future spread of infection, and choosing disease management strategies [8]. [9] proposed the radial basis feed forward neural network model and generalized regression for surface roughness prediction for face milling of Al 7075-T735. The Pearson correlation coefficients were also calculated to analyze the correlation between the five inputs (cutting speed, feed per tooth, axial depth of cut, chip's width, and chip's thickness) with surface roughness. [10] used radial basis function network to predict surface roughness and compared with measured values and the result from regression analysis. [11] considered three variables, that is, cutting speed, depth of cut and feed rate to predict the surface profile in turning process using radial basis function (RBF). Experiments have been carried out by [12] after end milling of steel C45 in order to obtain the roughness data and model of ANN for surface roughness predictions [13]. [14]developed a neuro-fuzzy system for Animal Feed Formulation (AFF) where percentage of each ingredient in poultry feed was determined by ANN and their different ingredients were combined with the help of Neuro-fuzzy and network.

Moreover, Fuzzy Inference System has been used for surface roughness prediction model for ball end milling operation. For the prediction of surface roughness, a feed forward ANN was used for face milling of aluminum alloy by [15] high chromium steel (AISI H11) by [16] and AISI 420 B stainless steel by [17].[17] proposed the analytical and artificial neural network models. [18] worked for selection of optimal machining parameters (that is, spindle speed, depth of cut and feed rate) for face milling operations in order to minimize the surface roughness and to maximize the material removal rate using response surface methodology (RSM) and perceptron neural network.

III. System Methodology

Data for the cassava diseases were gotten from domain expert. Crisp values are transferred into fuzzy values through fuzzification. The fuzzy inference mechanism uses predicted value to diagnose the cassava diseases as shown in Figure 1. The proposed mechanism was tested with the cassava diseases datasets. The mechanism was developed using MATLAB. Defuzzification converts the fuzzy set into crisp

values. The proposed method with predicted value technique can work more efficiently for diagnosis of cassava disease and also compared with earlier method using accuracy as performance metric.

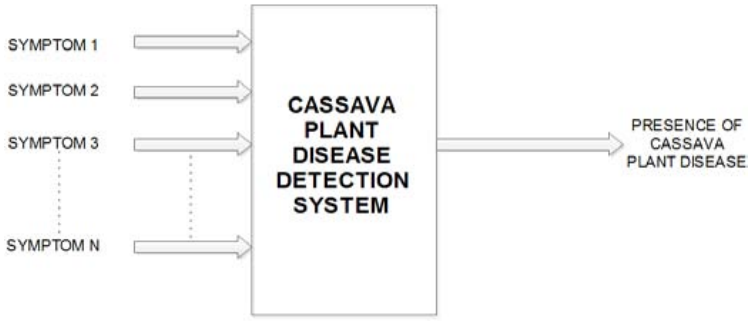

Figure 1 : Architecture for the Proposed Cassava Plant Disease for the Fuzzy Inference System

a) Diseases and their Symptoms

Different types of diseases affects the cassava plant, but in this paper, three of such diseases isput into consideration. These include Cassava Mosaic disease, Cassava Blight disease and Cassava Bacteria blight.

The symptoms for which these cassava diseases occur are: Chlorotic Leaf, Brown Lesions, Root Necrosis, Leaf Blight/spot, Distorted Leaf, Reduced Mishapen, Die-back and Gum Exudation. The value for the fuzzy variables are shown in Table 1.

Table 1 : Fuzzy variables and their Representations

Fuzzy variables	Representation of Fuzzy Variable(Linguistic terms)
Chlorotic leaf	No Chlorosis, Early Stages, Advanced Stages
Brown Lesions	No Presence, Early Stages, Advanced stages
Root necrosis	No symptom, Early Stage, Advanced Stages
Leaf blight/spot	No spots/Blights, Minor Spots/ blights,Major Spots/blights
Distorted Leaf	No Distortion, Lesser leave distortion, More leaf distortion
Reduced-Mishapen	No symptom, Stunted Growth
Die-Back	No symptom, Early Stage, Advanced Stages
Gum Exudation	No symptom, Early Stage, Advanced Stages

The input functions of the variables are represented in the membership function of the corresponding input and output.The data used were not too large, but the prediction was accurate to some extent.The output function is the result generated from the input function.

All the above-mentioned diseases have their own respective symptoms associated with them and were simulated using the Fuzzy logic inference system of Matric Laboratory (MATLAB) and implemented using C-Language Integrated Production System (CLIPS). This is a shell programming variety of PROLOG for developing expert systems. The first part of building an expert system is the knowledge acquisition. For this project, most knowledge was obtained from a human expert, an agriculturist who is specialized in detecting plant diseases. This knowledge was converted into a knowledge base using a set of rules and facts. The rules in this system represent symptoms and the actual results of every response. The proposed system uses the forward chaining inference mechanism, instead of backward chaining. The reason for using this mechanism is because when dealing with cassava plant disease detection, it is more important to collect information about the plant's symptoms first before making a decision. The forward chaining provides this competency.

IV. Result and Discussion

The fuzzy system is an in-built system that takes in multiple inputs into its membership function, these inputs are gotten from the data. The data, which in turn, has numeric values and assigned numbers and variables were used to classify and arrange these data. Sequelto this, some of these data were classified as categorical or numerical, in which there is adequate implementation.

A Fuzzy Inference System modelisrepresented in Figure 2. The systemcan either be loaded from the file. Loading the system from the file means inputting the name of the saved file where it has been kept. The input variables using membership functions are as shown in Figure 3, Figure 4 and Figure 5. The output model is shown in Figure 6. For the prediction, the rules used were 18 rules for the Cassava Mosaic and 27 rules for the cassava brown streak and 27 rules for cassava bacteria blight. These rules were sufficient for the prediction and the analysis shown in Figure 7. Rule Viewer Predicting Disease Severity is shown in Figure 8.

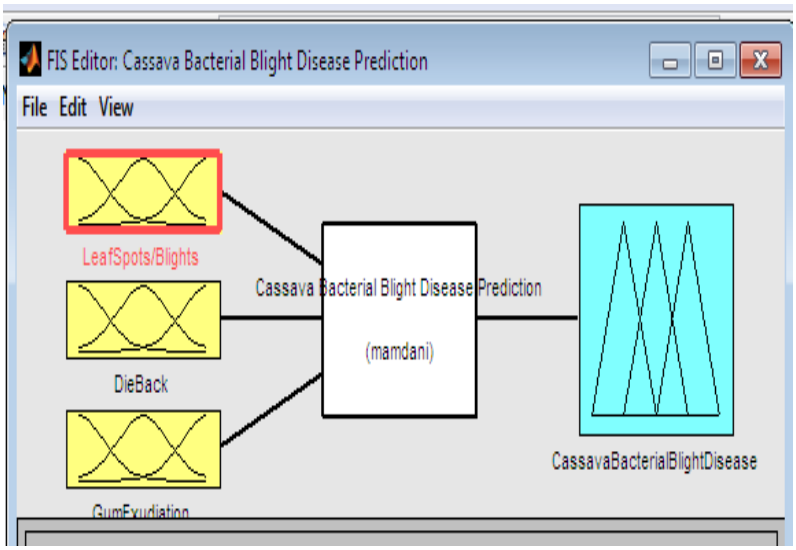

Figure 2 : FIS interface for Cassava Bacteria Blight

Input Variables

These variables are represented by triangular membership functions.

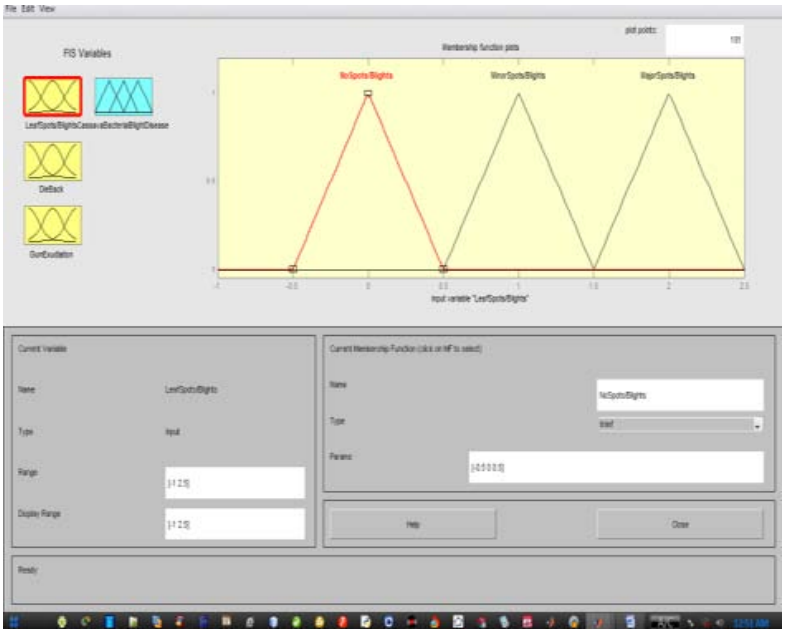

Figure 3 : Membership Function forLeaf Spot

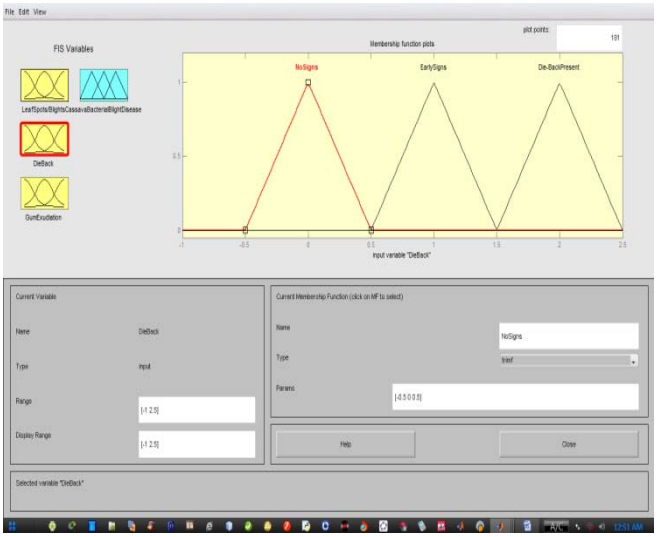

Figure 4 : Membership Function for Die-back

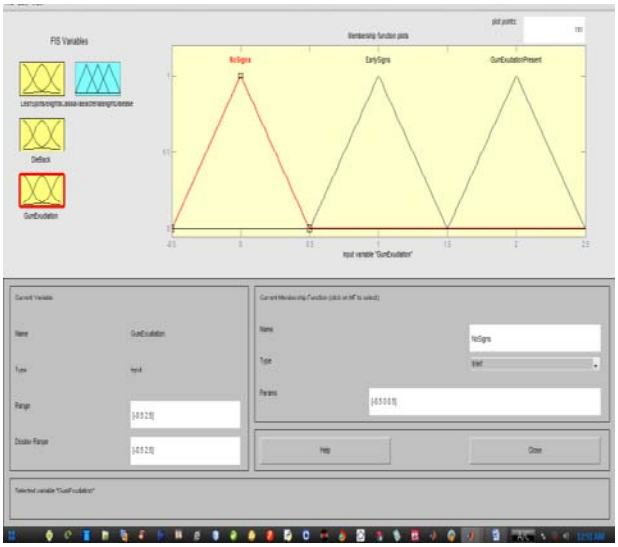

Figure 5 : Membership Function for Gum-exudation

Figure 6 : Membership function for Output Variable

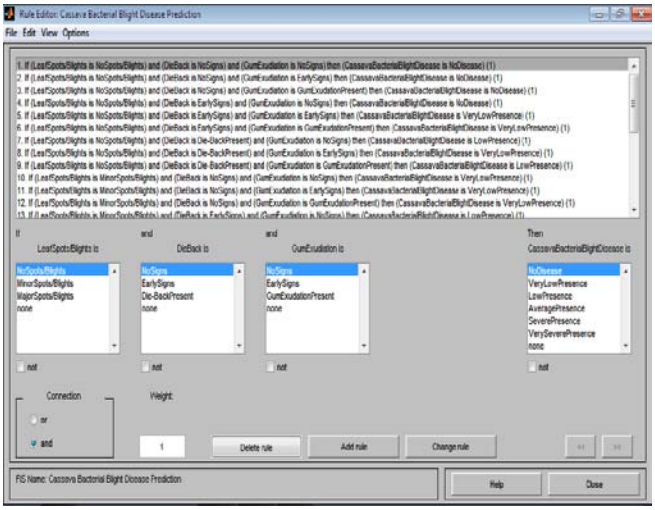

Figure 7 : FIS Output showing Rules Cassava Bacteria Blight

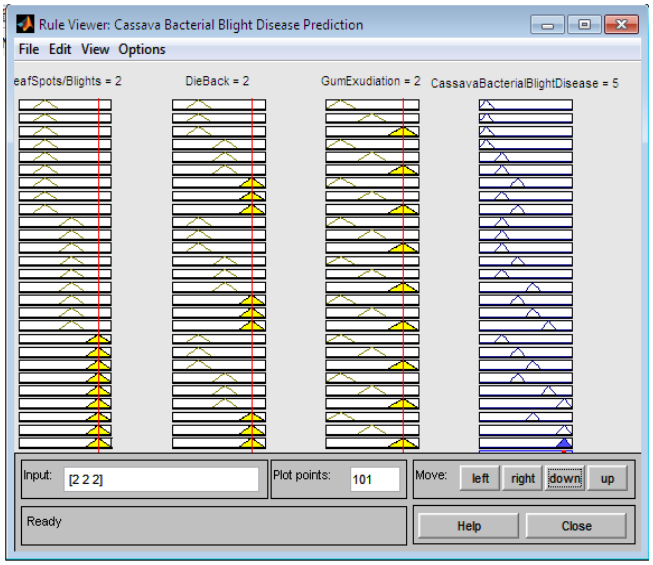

Figure 8 : Rule Viewer Predicting Disease Severity

Table 2 : The Diagnosis and Prediction of Cassava Mosaic Disease

Leaf Chlorosis	Leaf Distortion	ReducedMis-shapen	Output
No chlorosis	No distortion	No symptom	No disease
No chlorosis	No distortion	Stunted growth	No disease
No chlorosis	Lesser leaf distortion	No symptom	No disease
No chlorosis	Lesser leaf distortion	Stunted growth	Very low
No chlorosis	More leaf distortion	No symptom	Very low
No chlorosis	More leaf distortion	Stunted growth	Low
Early stages	No distortion	No symptom	No disease
Early stages	No distortion	Stunted growth	Very low
Early stages	Lesser leaf distortion	No symptom	Very low
Early stages	Lesser leaf distortion	Stunted growth	Low
Early stages	More leaf distortion	No symptom	Average
Early stages	More leaf distortion	Stunted growth	Severe

Advanced stages	No distortion	No symptom	Very low
Advanced stages	No distortion	Stunted growth	Low
Advanced stages	Lesser leaf distortion	No symptom	Low
Advanced stages	Lesser leaf distortion	Stunted growth	Severe
Advanced stages	More leaf distortion	No symptom	Average
Advanced stages	More leaf distortion	Stunted growth	Very severe

a) System Evaluation Using Domain Expert Diagnosis for Cassava Diseases

The system was tested with seven (7) cases for cassava diseases specified by the system and observed by the domain expert in line with his diagnoses. The diagnoses made by the system were classified as:

a. *Correct:* The system's diagnoses was identical with the pathologist's own diagnoses.
b. *Acceptable:* The system's diagnoses was different from that of the pathologist, but considered an acceptable alternative.
c. *Not complete:* The system's diagnoses was distinct enough for conclusion.

The accuracy of the system was calculated using the below formular.

Accuracy = (Number of Correct Diagnoses *100)/Total Number of Test Cases

The accuracy yields 71.4%.

V. Conclusion and Recommendations

The fuzzy inference system has broken the bounds of conventional programming, which is actually a function of its ability to adapt, adopt, adjust, evaluate, learn and recognize the relationship, behaviour and pattern of series of data set administered to it. It is tailored after the human reasoning and learning mechanism. This enables the FIS to handle and represent more complex problems than conventional programming. Fuzzy Inference System (FIS), fuzzy logic and expert system have helped in diagnosing and predicting diseases accurately.

Future work can be carried out with respect to more variables such as humidity and moisture content.

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Serotyping of *Salmonella Enterica* Isolated from Broiler Chicks in an Outbreak in Sudan

By Muna E. Ahmed & Marmar A. El Siddig

University of Khartoum, Sudan

Abstract- *Aims:* This study investigates the first emergence of S almonella outbreaks among 8.000 broiler chicks, of the ' Ross' breed, in Sudan. Mortality was observed in 25% of the t otal chicks; therefore, samples were taken for culturing, i dentification and characterization of the causative agent.

Study design: A total of 52 random samples, out of 2000 i nfected broiler chicks, were cultured and identified.

The study was undertaken in the laboratories of the Veterinary Research Institute, Ministry of animal resources and fisheries, Khartoum as well as Department of Botany, Faculty of Science, University of Khartoum, Sudan during March 2014- December 2014.

Results: All of the 52 isolates have shown colony characteristic t ypical to Salmonella spp. Result of the Vitek2 compact system s howed that isolate was typical Salmonella enterica. The p rimer pairs targeting invA and hilA genes successfully a mplified the extracted DNA giving the specific amplicons.

Keywords : *salmonella, serovars, salmonellosis, vitek P CR, sudan.*

GJSFR-C Classification : *FOR Code: 279999*

SEROTYPINGOFSALMONELLAENTERICAISOLATEDFROMBROILERCHICKSINANOUTBREAKINSUDAN

Strictly as per the compliance and regulations of :

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Muna E. Ahmed [α] & Marmar A. El Siddig [σ]

Abstract- *Aims:* This study investigates the first emergence of *Salmonella* outbreaks among 8.000 broiler chicks, of the 'Ross' breed, in Sudan. Mortality was observed in 25% of the total chicks; therefore, samples were taken for culturing, identification and characterization of the causative agent.

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Methodology: A total of 52 isolates were recovered from different organs of each chick. These isolates were characterized as *Salmonella enterica* (*S.enterica*) subspecies *enterica* using both conventional and automated methods. Vitek 2 Compact system was used as a fully automated method. Ten isolates were selected, as representative isolates, and were furthely identified using specific primers targeting each of *inv*A and *hil*A genes. The serotypes and phagetypes of each of the ten isolates were detected the examined number is very low.

Results: All of the 52 isolates have shown colony characteristic typical to *Salmonella* spp. Result of the Vitek2 compact system showed that isolate was typical *Salmonella enterica*. The primer pairs targeting *inv*A and *hil*A genes successfully amplified the extracted DNA giving the specific amplicons. Out of the ten representative *Salmonella enterica* isolates, nine showed the antigenic reaction of *S*. Enteritidis serovar and phagetype 3a while the tenth one was found identical to *S*. Typhimurium and phagetype 2.

Conclusion: The most pathogenic S*almonella* serovars, *S.* Enteritidis and *S.*Typhimurium were reported in the Sudan causing a serious economic risk in poultry farms.

Keywords: salmonella, serovars, salmonellosis, vitek PCR, sudan.

I. Introduction

Avian salmonellosis represents a group of acute and a chronic diseases caused by one or more members of genus *Salmonella* [1]. *S. enterica* is one of the most important public health pathogens [2] and may be acquired by the consumption of poultry products [3] and causes a number of significant diseases in poultry and humans. *S. enteric* is recently classified into more than 2500 serovars [4]. Most poultry infected by *Salmonella* spp become carriers; infection may also be fatal, depending on the particular serovar and the age of the bird at the time of infection [5].

Salmonellosis outbreak remains as a serious economic problem in countries where control measures are not efficient or in those areas where climatic conditions, favor the environmental spread of the microbes. The economic losses are chiefly due to morbidity, mortality, reduced growth rate, reduced feed conversion efficiency, drop in egg production, decrease fertility and hatchability.

In Sudan isolation of *Salmonella* was reported by different investigators. For example, Mamoun *et al*. [6] isolated 21 *Salmonella* strains from several poultry farms in three different states, all were found to be *S. enteritidis*. The isolation of *S. enterica sub enterica* serotype San-Diego from three goats (3.84%) at Omdurman Central Abattoir was reported [7]. Yagoub *et al.* [8] isolated *Salmonella* sp. from 1.43% of raw milk samples collected from different locations. Yagoub *et al*. [9] isolated *Salmonella Paratyphi A (S. Paratyphi* A) and *Salmonella Paratyphi B* (*S*. Paratyphi B) from 6% of the white cheese samples collected from retailer shops and restaurants in Khartoum and Omdurman. Yagoub [10] reported the isolation of *Salmonella* spp from 6.2% of the fish samples (gills, intestine, skin and muscles) collected from fish markets in Khartoum State. Forty-five *Salmonella* isolates (not serotyped) were isolated from carcasses, liver, spleen, intestinal contents of chicks from a poultry farm in El Obeid (unpublished data). Recently *Salmonella* Umbadah plus other 19 new serovars were reported from different sources at Khartoum [11; 12].

The Vitek system was originated in the 1970 as an automated system for identification and antimicrobial sensitivity test (AST) which automatically performs all the steps required for identification and AST of bacteria after primary inoculums have been prepared and standardized. This system allows kinetic analysis by reading each test every 15 minutes. The optical system combines multichannel fluorimeter and photometer reading to record fluorescence, turbidity and colorimetric signals [13].

Then, the aim of this study was to isolate and characterize the *Salmonella* spp. causing broiler chicks outbreaks.

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II. Material and Methods

Eight thousand (8,000) broiler chicks, of the 'Ross' breed, were bought for commercial benefits in March 2014. Due to mortality that was started at the first day, postmortem was done to investigate the gross lesions and taking samples from all organs. Samples were sent to Veterinary Research Institute, Ministry of animal resources and fisheries, Khartoum, Sudan for culturing, detection and identification of the causative agent.

a) Isolation and characterization of Salmonella

Salmonellae were isolated and identified according to the techniques recommended by the International Organization for Standardization described by Molla *et al*. [14]. Samples of broiler chicks including liver (n=10), intestine (n=10), heart (n=10), kidney (n=10), spleen (n=10), trachea and brain (one sample each) were each inoculated in nine ml of sterilized buffered peptone water. The inoculated buffered peptone water was incubated at 37°C for 24 hours and the resulting suspensions were cultured on selenite broth medium and then purified on nutrient, MacConkey and XLD media (Hi media, India). Cellular, colony morphology and biochemical characteristics of each isolate were tested [15].

b) Vitek 2 Compact Automated System

Ten representative isolates, selected from each of the examined organs, were furtherly characterized using full automated system Vitek 2 compact (bioMerieux) to confirm the species *S.enterica*. The Gram Negative card that used in Vitek2 compact was based on established biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities and resistance [16; 17; 18]. The GN card used contained a total of 47 wells representing 47 different biochemical tests and one negative control well. Identification was done according to the manufacturer's guidelines.

c) Molecular identification of Salmonella isolates

PCR primers, targeting each of *inv*A (F: 5´ GTG AAA TTA TCG CCA CGT TCG GGC AA3´ and R: 5´ TCA TCG CAC CGT CAA AGG AAC C 3´ [19] and *hil*A (F: 5´-CTG CCG CAG TGT TAA GGA TA-3´ and R: 5´-CTG TCG CCT TAA TCG CAT GT-3´ [20] *Salmonella* specific genes, were evaluated to detect the isolated *Salmonella*. DNA from each isolate (n=52) was extracted according to the boiling – centrifugation method [21]. A single colony of a pure nutrient agar culture was grown overnight at 37°C in 1.0 ml Luria–Bertani broth. Bacterial cells were precipitated by centrifugation at 13,000 rpm for 5 min. in a micro-centrifuge (MSE, MSBo1o.cx2.5, Sanyo, UK). The supernatant was discarded and the pellet was re-suspended in 500µl deionized distilled water. The suspension was boiled for 10 min. in a water bath then immediately cooled on ice. Extracted DNA was then stored refrigerated at 4°C until used as a template for PCR amplification.

The extracted DNA was amplified by an established PCR technique [22]. PCR amplification reactions were carried out in 25 µl total volume of PCR mixture containing 5 µl of template DNA, 12.5µl of the PCR master mix (Promega) (50 unit/ml *Taq* DNA polymerase in an appropriate reaction buffer {pH 8.5}, 400 µM each dNTPs and 3mM $MgCl_2$) and 0.1 µM of each of primer pair. DNA was amplified according to reaction conditions published for each primer pair in a thermal cycler (Techne/ Flexigene - biotech). The cycling conditions were as follow: an initial incubation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 64°C (*inv*A) or 62°C (*hil*A) for 60 seconds, extension at 72°C for 60 seconds, and final extension period for 7 minutes at 72°C.

Appearance of the target band specified for each primer set on the 1.2% agarose gel under specified gel electrophoresis conditions is considered as a positive amplification product.

d) Salmonella Serotyping and Phage typing

Ten presumptive *Salmonella* isolates (selected based on their biochemical reactions and molecular identification) were shipped to the Public Health Agency, Office International des´ Epizooties (OI´E) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada for serotyping and phagetyping. The antigenic formulae of Popoff and Le Minor [23] were used to name the serovars. Phagetyping was performed using the standard phagetyping technique described by Anderson and Williams [24].

III. Results

a) Conventional biochemical tests identification

The average cumulative mortality percentage in the 8,000 broiler chicks, during the period from 1 to 8 days of age, was 25%. A total of 52 bacterial isolates, cultured from different internal organs, were recovered on selenite broth, Nutrient, MacConkey and XLD media. All of the isolates were Gram negative and have shown colony characteristic typical to *Salmonella* spp. The isolates were positive for citrate and methyl red tests and they were negative for indole, Voges-Proskauer and urease tests.

b) Vitek 2 Compact Automted System

Result of the Vitek 2 compact system showed that the isolates were typical *Salmonella enterica*.

c) Molecular identification

DNA extracted from the isolates were used to evaluate the specificity of two primer sets to detect *Salmonella* sp. The primer pairs targeting *inv*A and *hil*A genes successfully amplified the extracted DNA giving the specific amplicons for each primer (Fig. 1).

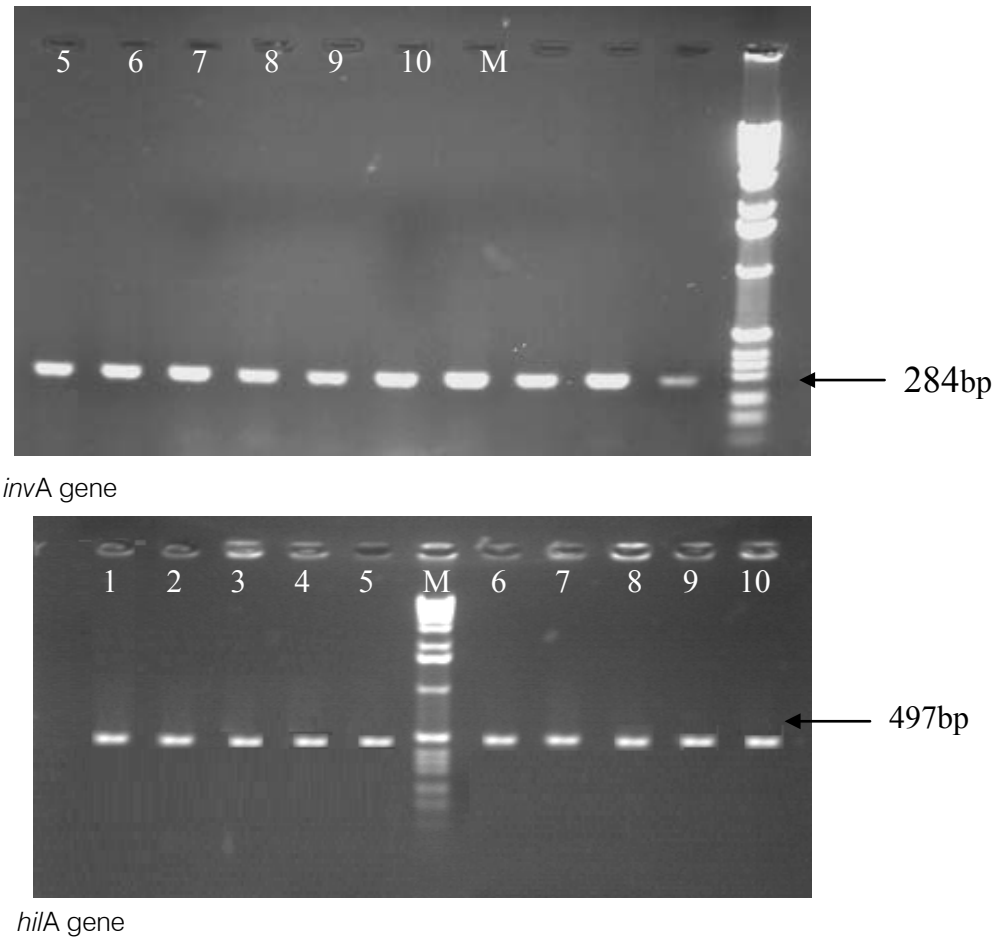

Figure 1 : PCR amplification products detected for the two primer sets. Lanes 1 - 10 represent *Salmonella* isolates, M = 100bp DNA ladder

d) Serotyping and Phagetyping

Serotyping test showed that all of the tested isolates (*n* =10) were members of *S. enterica* subspecies enterica. Results in Table 1 show that nine of the ten isolates reported here belonged to serovar Enteritidis (9,12:g,m:-) and one isolates was serotyped as *S.* Typhimurium (4,5:i:1,2). All of the nine Enteritidis isolates were phagetype 3a while the Typhimurium isolate was phagetype 2.

Table 1 : *Salmonella* serotyping and phagetyping results

Salmonella isolate No.	Antigenic formula	Serovar	Phagetype
1	9,12:g,m:-	Enteritidis	3a
2	9,12:g,m:	Enteritidis	3a
3	4,5:i:1,2	Typhimurium	2
4	9,12:g,m:	Enteritidis	3a
5	9,12:g,m:	Enteritidis	3a
6	9,12:g,m:	Enteritidis	3a
7	9,12:g,m:	Enteritidis	3a
8	9,12:g,m:	Enteritidis	3a
9	9,12:g,m:	Enteritidis	3a
10	9,12:g,m:	Enteritidis	3a

The mortality rate of 8.000 chicks was 25% (2000). The other chicks which were 75 % (6000) survived under treatment using Gentadox (Avico) that contain 200mg of gentamyicin sulphate and 125 mg of doxycycline hydrochloride.

IV. Discussion

Salmonellosis is a common bacterial infection in human. Illness caused by different *Salmonella* spp. can range from a mild to severe gastroenteritis and in some people, invasive disease, which can be fatal. Long term consequences such as reactive arthritis can also result from *Salmonella* infections [25]. Symptoms of *Salmonella* outbreak was observed on 25% of the commercial broiler chicks. It is likely due to the fact that chicks are not fully immune competent when they are below 2 weeks of age because of a lower percentage of CD4+CD8- in the thymus; CD4-CD8+ and CD4+CD8+ in the spleen [26]. Many researchers have reported that salmonellas is outbreaks in human were associated with poultry meat or eggs [27; 28]. Vertical transmission of infections from breeding hens to progeny has been an important aspect of the epidemiology of *Salmonella* species infections within the poultry industry [29; 30].

In this study *Salmonella* were identified in 52 intact samples, selected from 2000 symptomatic chicks, ten of them were serotyped as S. Enteritidis and *S.* Typhimurium are by far the two dominating serotypes isolated from poultry and poultry products [31; 32] and these two serotypes are also the most frequently isolated serotypes in humans [33; 34]. A number of *Salmonella* outbreaks reported in the world are a result of injudicious introduction of infected birds [35-38]. According to FAO/WHO reports [39], most foodborne *S.* Enteritidis infection is associated with the consumption of raw eggs and foods containing raw eggs. In the European Union-wide baseline study of *Salmonella* in commercial broiler flock, 2005-2006, the observed prevalence of positive flocks was 23.7 % while the member-state specific rates varied from 0.0 to 68.2 %. A total of 11.0% of the broiler flocks were estimated to be positive for *S.* Enteritidis and/or *S.* Typhimurium. The Member State-specific observed flock prevalence of *S.* Enteritidis and/or *S.* Typhimurium varied from 0% to 39.3% [40]. In an experimental infection done by Akhtar, *et al.* [41] showed the pathogenisity of *S.* Enteretidius phagetype 3a in a newly hatched white leghorn chicks.

V. Conclusion

The most pathogenic species of *Salmonella, S.* Enteritidis and *S.* Typhimurium were reported in the Sudan causing a serious economic risk in poultry farms.

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The Prevalence of Intestinal Helminthiasis in Primary School Children in Isuochi Umunneochi Local Government Area, Abia State, Nigeria

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Abstract- Prevalence of intestinal helminth infections in primary schools in Isuochi town, Abia State Nigeria was surveyed in two randomly selected primary schools, between April and September 2012. Stool samples of 200 pupils (110 males, 90 females), aged 6-9 years, were examined microscopically by using wet mount (normal saline) and concentrated saturated sodium chloride floatation techniques. Seven intestinal helminths, Ascarislumbricoides, Hookworm, Trichuristri- chiura, Strongyloidesstercoralis, Enterobiusvermicularis, Schistoso-mamansoni and Taeniaspp, were identified with 150(75%) of the 200 pupils infected with one or a combination of the worms. Hookworm had the highest prevalence (37.84%) followed by A. lumbricoides (24.32%), T. trichiura(14.86%), E. vermicularis(8.11%), S. stercoralis and T. spp have (6.76%) infection rate respectively while S. mansonihas the lowest rate of infection (2.70%).

Keywords : *children, helminthiasis, intestinal.*

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Azoro A. V. [α], Awurum I. N. [σ], Nwoke B. E. B [ρ], Chinaka A. A. [ω], Tony-Njoku. R. F. [¥], Egeruoh A. S[§] & Nwakor, F .N [χ]

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Keywords: children, helminthiasis, intestinal.

I. Introduction

The health of school age children in developing countries is a concern that has received increasing attention in the recent past, following high morbidity rates due to parasitic disease which are preventable (Bundy and Guyatt, 1995). Much of this morbidity has been attributed to parasitic helminth infections (Etim and akpan 1999, Etim et al, 2002).

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The public health and socio-economic consequence of intestinal helminthiasis are of considerable global concerns particularly in the rural communities of the developing countries where malnutrition and other factors complicate the impact of the infection. Between 500 and 1000 million people were estimated to be infected with parasites with direct life cycles 35 years ago (PETERS 1978); meanwhile the number has certainly considerably increased. EDUNGBOLA (1988-90) estimated that 15 million Nigerians are suffering from ascariasis alone, while there are several thousand with hookworm, trichuriasis, enterobiasis, strongyhoidiasis, tapeworm infections and others.Apparently, the epidemiology of human intestinal parasites is vastly recorded in Nigeria. In most cases hospital records have become an increasingly popular method of determining prevalence of these diseases (Cowper 1967; Obiamiwe 1977; Reinthaler et al 1988).

Various prevalence rates on infection of these helminths in school children in different parts of Nigeria have been reported by several workers. Okpara et al (2007) for instance, obtained prevalence rate of 65.6%, 35.2% and 14.8% for Ascarisspp, Hookworm spp and Trichurisspp respectively. And Etim et al (2002), obtained a prevalence rate of 53.2%, 31%, 27.0% and 5.5% for *Ascarissp*, *Ancylostomasp*, *Trichurissp* and *Schistosoma mansoni* respectively, all from primary school children aged 5-16 years in Owerri and Calabar, Nigeria. The prevalence of these helminths varies not only from one locality to the other, but also among individuals, age, standard of sanitation, socio-economic status of parents, wih children of parents in the low income group having the highest prevalence of infection and sex with males being more infected than females ofinterest in intestinal helminthic infection is multiple infection or poly parasitism, occurring in various combinations and rate of infection but with Ascaris-Hookworm-Trichuris "Traid" combination as the most regular combination.

Factually, many studies on intestinal helminthiasis of school children have been carried out in many parts of the country. It is still important to carry similar studies in different other parts of the country at different times in view of the changing patterns of parasitic infections. The present study aims at the

identification of various intestinal helminth parasites, which infect primary school children, to determine the overall prevalence of infection and the pattern of infestation in relation to age and sex of the children, and reports the results of the investigation on intestinal helminthiasis in school children in Isuochi Primary Schools, Abia State, Nigeria.

II. Materials and Methods

The study was carried out in Isuochi. Two hundred school children, aged 6-9 years in two randomly selected primary schools in Isuochi Town, Abia State, were investigated for their intestinal helminthic infections between April and September 2012. The schools are

School

Amuda Town Primary School Isuochi S1

Isuochi Central School Isuochi S2

a) Collection and examination of feacal samples

Wide mouthed specimen bottles were given to the randomly selected pupils who were asked to return them the following morning with feaces for examination. The name (optioned) age and sex of each child were labeled on the respective specimen collected. The specimens were taken to the laboratory for examination with a Nikkon compaind microscope using X10 and X40 objectives. The normal saline (wet mount) and concentrated saturated sodium chloride floatation techniques according to Nera and Brown (1994) Chees brough (1999) were used for the analysis of the feacal samples for helminth ova and larvae. On collection of feacal samples from the pupils, each pupil was interviewed on some of the following points: parents occupation, foot wear habits, domestic animals reared, regularity of the children's de-worming and availability and type of toilet facility etc. Data collected wer analyzed by means of descriptive statistics such as frequency tables, percentages and Chi square.

III. Results

The result of the study showed that School samples from a total of 200 primary school pupils comprising 110 males and 90 females, aged between 6 and 9 years, were examined for intestinal helminth infections. Seven intestinal helminth, *Ascarislumbricoides*, *Hookworm*, *Trichuristrichiura*, *Strongyloidesstercoralis*, *EnterobiusvermicularisSchistosomamansoni* and *Taeniaspp* were identified. Of the 200 pupils examined, 150 (75.0%) were infected with one or a combination of the worms with hookworm having the highest prevalence rate (28.0%), followed by *A. Lumbricoides* (24%) *I. Trichiura* (14.7%), *E. Vermicularis* (8.0%) *S. Stacoralis* and *Taeniaspecies* (6.7%) respectively, whereas S. mansoni had the least rate of infection (2.7%). The results are shown in table 1. The findings of the highest occurance of hookworm infection more than *A. lumbricoides* is rare in a study like this.

The prevalence of infection among the schools ranged between 7.0% (S1) and 78% (S2). There was no significant difference in the prevalence of infection between schools. ($P>0.05$).

Table 2 shows that the overall prevalence of infection of the helminthes was highest in pupils aged 9 years (80%) and lowest in pupils aged 7 years (640%). The prevalence of *A. lumbricoides* and Hookworm were highest in pupils of age 7, 8 years and 6 years respectively and both lowest in pupils of age 9 years. There was no statistical significant difference in infection prevalence with age ($P>0.05$). the table further shows that *T. trichiura S. mansoni* were also highest in age 7 respectively.

Table 2 also shows that out of 200 pupils examined 110 (55.0%) males and 90 (45.0%) were males and females respectively, of these 88 (80%) males and 62 (68.9%) females were infected. The difference not significant ($P>0.05$). The prevalence of Hookworm and *A. lumbricoides* were higher in females than in males while that of *T. trichiura* where higher in males. The difference was not significant ($P>0.05$). This is in agreement with the discoveries of Okpara et al 2007. Of the 150 infected subjects, 22 (14.7%) had multiple intestinal helminth infections with 14 (9.3%) and 8 (5.3%) subjects having bouble and triple infections respectively. *Ascarislumbricoides* occurred mostly with other helminth, Ascaris + hookworm, and Ascaris + hookworm + Trichuris were the most common occurring combinations. These results are shown in table 3.

IV. Discussion

Ova of seven intestinal helminthes, hookworm, *Ascarislumbricoides*, *Trichuristrichiura*, *Strungyloidesstercoralis*, *Enterobiusvermicularis*, *Schistosomamansoni* and *Taeniaspp* were recorded with 150 (75%) of the 200 school children positive for one or more types of helminth. The overall prevalence of infection (75%) when compared with reported studies of previous studies in other parts of the country (Mafiana, 1995, Ukpai and Ugwu, 2003) agrees with their findings but is high when compared with (Okpara 2007, and Dada et al 1993), suggestive of poor personal hygiene awareness and environmental sanitation in the study area and indefinite communal control efforts. Previous studies had also attributed the high endemicity to poor environmental and personal hygiene, shortage of good water supply and indiscriminate defecation. Hookworm and *Ascarislumbricoides*, in contrast to the other helminths had the highest prevalence of infection probably because their Ova can live in soil for years and are resistant to environmental pressures.

However, the prevalence of ascariasis and hookworm infections decreased with age 9 groups

probably indicating maturity in age and awareness of the existence of such diseases. Poor parental hygiene, supervision, voracious eating habit and activities linked with contaminated with infected intestinal helminth infections s common in school children in Nigeria. In this study, through sex differences in the prevalence of intestinal helminth infections was not significant,. Males were more infected (72.7%) as against females (66.6%). Many of the children infected with this helminth are from homes in which goats, sheep or rabbits are domestically reared and their feaces used as nature in domestic vegetable gardens.

Mixed infection due to Ascaris, hookworm and Trichuris often described as "Ubiquitous triod" Ascaris and hookworm was common, which is in consonance with the findings of mba and Amadi (2001), Ukpai and Ugwu (2003) Opara et al (2007).

From an epidemiological perspective therefore the study underlying the fact that indiscriminate defecation, food and feeding habits amenities and awareness of the mode of transmission as well as low level of sanitation of the study areas are among the principal factors enhancing transmission of helmthiasis in the area studied. This situation calls for effective control measures in the community. Perhaps, this can be achieved through community health education campaign aimed at influencing the attitudes and behaviours of the population at risk regarding the consumption of well cooked meat, maintaining a high standard of sanitation and treating diagnosed cases.

A similar recommendation has been made by Ukoli (1990). Prevention of these intestinal helminth infections is possible by restricting sheep, goat and cattle from straying, avoiding ingestion of predisposed food, avoiding use of human and animal excreta as fertilizer in vegetable gardens, avoiding bathing in infected streams and lakes and by maintaining personal hygiene.

Table 1 : The prevalence of intestinal helminths in school children in selected primary schools in Isuochi

School Code	No. of pupils Examined	No. infected (%)	Helminths identified/% prevalence A.1 H (%)	T.t (%)	S.S (%)	E.V (%)	S.M (%)	I SPP (%)	(%)
SI	100	72(72%)	20 (27.8%)	25 (34.7)	9 (12.5)	5 (6.9)	5 (6.9)	0 (0.0)	6 (8.3)
S2	100	78(78%)	16 (20.5)	31 (28.0%)	13 (16.7)	5 (6.4)	7 (9.0)	4 (5.1)	4 (5.1)
TOTAL	200	150(75%)	36 (18.0)	56 (28.0)	22 (11.0)	10 (5.0)	12 (6.0)	4 (2.0)	10 (5.0)

Source; Field survey 2012

Table 2 : Prevalence of intestinal helminth infections in relation to Age and Sex of pupils

Age Range (yrs)	No examined	No Infected %	A coides %	Lumbri %	Hook worm %	T.t %	S.s %	E.v %	S.m %	T.spp %
6	m20	18(90.0)	5(27.8)	9(50.0)	0(00.0)	3(16.7)	0(00.0)	2(11.1)	2(11.1)	
	F14	11(78.6)	3(27.3)	3(27.3)	0(00.0)	1(9.1)	0(00.0)	0(00.0)	2(11.2)	
	T34	29(85.3)	8(27.6)	12(41.4)	0(00.0)	4(13.8)	0(00.0)	2(6.9)	4(13.8)	
7	m42	31(73.1)	6(19.4)	12(38.7)	9(29.0)	1(3.2)	7(22.6)	0(00.0)	1(3.2)	
	F34	28(823)	2(7.1)	6(21.4)	5(17.9)	3(10.7)	3(10.7)	0(00.0)	1(3.6)	
	T 76	59(77.6)	8(15.7)	18(30.5)	14(23.7)	4(6.8)	10(16.9)	0(00.0)	2(3.9)	
8	m29 37	29(78.4)	10(34.5)	18(62.1)	2(6.9)	1(3.4)	0(00.0)	1(3.4)	3(10.3)	
	F26 33	20(60.6)	6(30.0)	6(30.0)	0(00.0)	1(5.0)	0(00.0)	1(5.0)	1(5.0)	
	T 70	49(70.0)	16(32.7)	2449.0)	2(4.1)	2(4.1)	0(00.0)	2(4.1)	4(8.2)	
9	m 11	10(90.9)	2(20.0)	0(00.0)	4(40.0)	0(00.0)	2(20.0)	0(00.0)	0(00.0)	
	F 9	3(33.3)	2(66.7)	2(66.7)	2(66.7)	0(00.0)	0(00.0)	0(00.0)	0(00.0)	
	T 20	13(65.0)	4(30.8)	2(15.4)	6(46.2)	0(00.0)	2(15.4)	0(00.0)	0(00.0)	
Total	m 110	m(88(80.0)	23(26.1)	39(44.3)	15(17.0)	5(5.7)	9(10.2)	3(3.4)	6(6.8)	
	F 90	f(62(68.9)	13(30.0)	17(27.4)	7(11.3)	5(8.1)	3(4.8)	1(1.6)	4(6.4)	
	T 200	T-150	36(24.0)	50(37.3)	22(14.7)	10(6.7)	12(8.0)	4(2.7)	10(6.7)	

*% Prevalence in parenthesis. *p>0.05*

m = male f = female T = total

Table 3 : Poly parasitism in school children in selected towns in Umunneochi L.G.A.

Parasite combination	No. infected	%infection
Ascaris + Hookworm	8	5.3
Ascaris + *Trichiura*	4	2.7
Ascaris + Hookworm + *Trichiura*	3	2.0
Ascaris + *Strongyloidesstercorlis*	2	1.3
Hookworm + T.spp	2	1.3
Hookworm + *Schystogomamansoni*	1	0.7
Ascaris + Hookworm + *Enlerobiusvermical*aris	2	1.3

Total number infected with intestinal helminths = 150.
% Prevalence of infection in parenthesis based on 150 infected.

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TECHNIQUES FOR WRITING A GOOD QUALITY RESEARCH PAPER:

1. Choosing the topic: In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

2. Evaluators are human: First thing to remember that evaluators are also human being. They are not only meant for rejecting a paper. They are here to evaluate your paper. So, present your Best.

3. Think Like Evaluators: If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

4. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

5. Ask your Guides: If you are having any difficulty in your research, then do not hesitate to share your difficulty to your guide (if you have any). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work then ask the supervisor to help you with the alternative. He might also provide you the list of essential readings.

6. Use of computer is recommended: As you are doing research in the field of Computer Science, then this point is quite obvious.

7. Use right software: Always use good quality software packages. If you are not capable to judge good software then you can lose quality of your paper unknowingly. There are various software programs available to help you, which you can get through Internet.

8. Use the Internet for help: An excellent start for your paper can be by using the Google. It is an excellent search engine, where you can have your doubts resolved. You may also read some answers for the frequent question how to write my research paper or find model research paper. From the internet library you can download books. If you have all required books make important reading selecting and analyzing the specified information. Then put together research paper sketch out.

9. Use and get big pictures: Always use encyclopedias, Wikipedia to get pictures so that you can go into the depth.

10. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right! It is a good habit, which helps to not to lose your continuity. You should always use bookmarks while searching on Internet also, which will make your search easier.

11. Revise what you wrote: When you write anything, always read it, summarize it and then finalize it.

12. Make all efforts: Make all efforts to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in introduction, that what is the need of a particular research paper. Polish your work by good skill of writing and always give an evaluator, what he wants.

13. Have backups: When you are going to do any important thing like making research paper, you should always have backup copies of it either in your computer or in paper. This will help you to not to lose any of your important.

14. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several and unnecessary diagrams will degrade the quality of your paper by creating "hotchpotch." So always, try to make and include those diagrams, which are made by your own to improve readability and understandability of your paper.

15. Use of direct quotes: When you do research relevant to literature, history or current affairs then use of quotes become essential but if study is relevant to science then use of quotes is not preferable.

16. Use proper verb tense: Use proper verb tenses in your paper. Use past tense, to present those events that happened. Use present tense to indicate events that are going on. Use future tense to indicate future happening events. Use of improper and wrong tenses will confuse the evaluator. Avoid the sentences that are incomplete.

17. Never use online paper: If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

18. Pick a good study spot: To do your research studies always try to pick a spot, which is quiet. Every spot is not for studies. Spot that suits you choose it and proceed further.

19. Know what you know: Always try to know, what you know by making objectives. Else, you will be confused and cannot achieve your target.

20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After 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 to 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 in 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 criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of 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 the 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 evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

Title Page:

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

Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing 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 approach 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. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than 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 maintain the initial two items to no more than one ruling each.

- Reason of the study - 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 quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness 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 to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled 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 with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose 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 sound written Procedures segment allows a capable scientist to replacement 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 for the least amount of information that would permit another capable scientist to spare 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 object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text 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:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not 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 - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in 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 a 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. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise 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 in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to 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 part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a 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 implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly 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 with prospect, and let it drop at that.

- Make a decision if each premise is supported, 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 the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One 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 available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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- To guard yourself and others from possible illegal use please do not permit anyone right to use to your paper and files.

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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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