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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

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## Full Length Research Paper

# Proximate composition, antinutritional contents and physicochemical properties of breadfruit (*Treculia africana*) and cowpea (*Vigna unguiculata*) flour blends fermented with *Lactobacillus plantarum*

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This study investigates proximate composition, antinutritional contents and physicochemical properties of breadfruit (*Treculia africana*) and cowpea (*Vigna unguiculata*) flour blends fermented with pure strains *Lactobacillus plantarum* (FO-12) for the purpose of developing weaning food for the management of protein-energy malnutrition (PEM). Breadfruit and cowpea flour blends were prepared in various ratios and then fermented using *Lactobacillus plantarum*. Proximate composition, phytic acid, hydrogen cyanide and oxalate contents were determined. pH and titratable acidity were also determined. A significant increase ( $p < 0.05$ ) was observed in the ash, protein and fat contents of the fermented flour blend as the amount of cowpea flour increases in the formulation; whereas crude fibre and carbohydrate contents significantly decreased ( $p < 0.05$ ). Similarly, moisture, protein and fat contents significantly increased ( $p < 0.05$ ) with fermentation. Significant reductions were observed in crude fibre and carbohydrate contents after fermentation. Fermentation process caused a significant decrease ( $p < 0.05$ ) in the oxalate, phytate and hydrogen cyanide contents of the breadfruit flour blends. The changes observed in the fermented flour blends agreed with significant decreases recorded for pH and increases in titratable acidity. Fermentation improves the nutritional composition of breadfruit-cowpea flour blends for possible use as complementary foods for infants providing PEM management.

**Key words:** Breadfruit-cowpea flour blends, fermentation, *Lactobacillus plantarum*, weaning food, physico-chemical properties.

## INTRODUCTION

African breadfruit (*Treculia africana*) from the mulberry family *Moraceae* is an important food crop in Nigeria (Ejiofor et al., 1988). It is widely cultivated in the southern

states of Nigeria (Badifu and Ubor, 2001; Ugwu et al., 2001) and generally regarded as the poor man's substitute for yam (*Dioscorea esculenta* and *Dioscorea*

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*cayenensis*). This is due to the fact that it is used in several traditional food preparations of yam, and also cost less than one third the price of yam at the market (Mayaki et al., 2003). Breadfruit contains about 1.34% protein, 0.31% fat, 27.82% carbohydrate, 1.5% fibre and 1.23% ash (Wang et al., 2011). It is also rich in calcium, phosphorus, iron, potassium, carotene and vitamin B (Wang et al., 2011). Breadfruit powder or flour is reported to contain about 76.7% carbohydrate (Morton, 1987), 17.1% protein, 11% fat, 3.0% ash and 0.1% crude fiber (Akubor et al., 2000).

The presence of anti-nutritional factors limits the use of this crop. However, they could be eliminated or reduced by some processes such as soaking, dehulling, germination and fermentation (Khokhar and Chauhan, 1986). The study of Onweluzo and Nnamuchi (2009) reveals that fermentation and steaming enhances the detoxification of breadfruit flour. But fermentation as a method of processing and preserving breadfruit, as obtains in the Pacific Islands, is quite unpopular in most areas where it has been introduced (Adekanmi et al., 2012). Protein-energy malnutrition (PEM) has resulted in high level of infants and children mortality in Nigeria, especially among the low income earners. Cowpea is an important food legume indigenous to Africa (Khalid et al., 2012). It provides more than half the plant protein in human diets (Rachie, 1985). Cowpea has been reported to contain about 20.07 to 24.60% protein, 46.84 to 53.63 starch (carbohydrate), 1.77 to 1.96% fat and 4.27 to 4.96% fibre (Preet and Punia, 2000), as well as 3.50% ash (Darfour et al., 2012). The black-eyed cowpea (*Vigna unguiculata*) used for this research is an important food legume in Nigeria.

Composite meals have been developed from African breadfruit and tubers such as sweet potatoes, yam and cocoyam (Akubor, 1997; Oladeji and Akanbi, 2011) and cereals like sorghum (Onweluzo and Nnamuchi, 2009) but enrichment of breadfruit with cowpea which has the potential of alleviating protein-energy malnutrition among children in Nigeria has not received considerable attention. Due to the high dependence on breadfruit as a staple food in tropical Africa, coupled with the low nutritive value of the commodity, it is imperative to investigate simple processing method that can improve the nutrition qualities of the crop.

Although, fermentation improves the nutritive value of cowpea, but the process is not popular in Nigeria. Fermentation of breadfruit with cowpea could be a useful process of improving the health of vast numbers of infants and children at low cost especially in the rural areas. This might offer a significantly cheap and sustainable food process that will reduce micronutrient deficiency. Our previous study has been on the effect of spontaneous fermentation on nutrient and anti-nutrient composition of breadfruit and cowpea blend flours. In this study, the proximate, antinutritional and physicochemical properties of African breadfruit and cowpea flour blends

that are fermented with pure strain of *Lactobacillus plantarum* (FO-12) isolated from spontaneous fermentation of breadfruit were investigated.

## MATERIALS AND METHODS

### Source of raw materials

Freshly harvested, matured but un-ripened breadfruit (*Treculia africana*) and dried cowpea (*Vigna unguiculata*) were purchased from a local market in Ibadan, south-west area of Nigeria. These raw materials were packaged in low density polyethylene bag and then transported to the Laboratory for processing and analyses.

### Preparation of breadfruit flour

Breadfruits were washed, manually peeled and diced into sizes. The diced fruits were blanched in boiling water (100°C) for 10 min., dried in air-oven at 65°C for 24 h., milled using Hammer Mill (Brook Crompton, Huddersfield, England) and then sieved through 600 µm aperture size to obtain fine flour (Figure 1).

### Preparation of cowpea flour

About 100 g of cowpea was sorted and cleaned to remove extraneous materials and defective seeds. The seeds were soaked in distilled water for 25 min and then dehulled using mortar and pestle. The dehulled seeds were washed, dried at 65°C for 24 h in air-oven, milled and then sieved through 600 µm aperture size to obtain fine flour (Figure 1).

### Preparation of breadfruit-cowpea flour blends (composites)

Breadfruit-cowpea flour blends were composited in the ratios 100:0 breadfruit flour to cowpea flour blend (TvA), 90:10 breadfruit flour to cowpea flour blend (TvB), 80:20 breadfruit flour to cowpea flour blend (TvC), 70:30 breadfruit flour to cowpea flour blend (TvD), 60:40 breadfruit flour to cowpea flour blend (TvE) and 50:50 breadfruit flour to cowpea flour blend (TvF) (Ojokoh et al., 2013) as outlined in Figure 1.

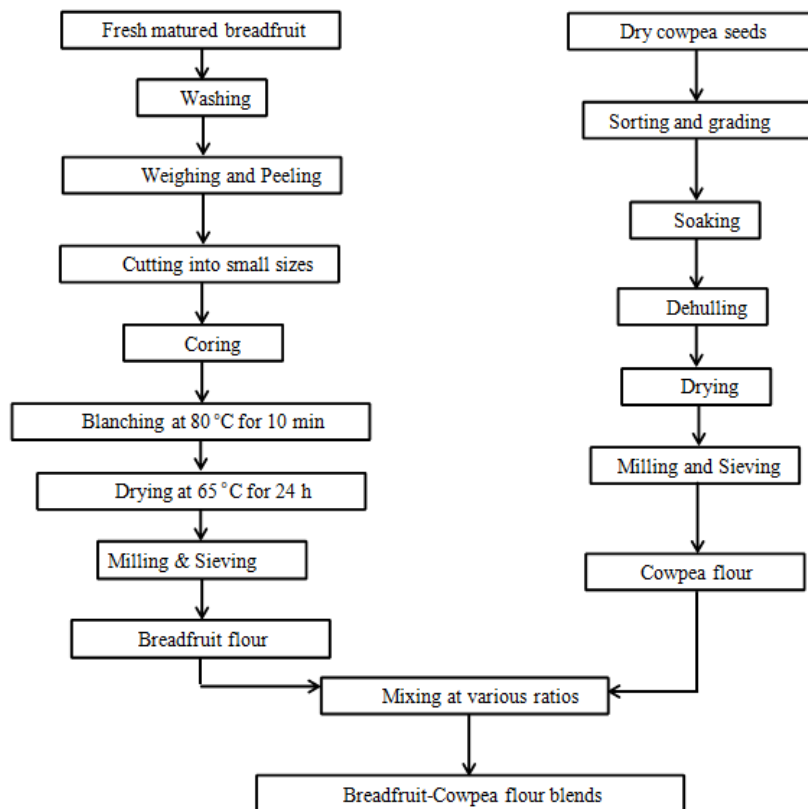
## Fermentation process

### Bacterial strains used for fermentation

The strain of *Lactobacillus plantarum* (*L. plantarum*, FO-12) used for the fermentation was isolated from previous study on spontaneous fermentation of breadfruit (Ojokoh et al., 2013). The strain was selected after morphological, phenotypic and molecular characterisation as described by Sawitzki et al. (2007). The isolated strain was preserved in microbial vials.

### Fermentation of the breadfruit-cowpea flour blends

The *L. plantarum* (FO-12) was first grown in MRS broth (Merck, Darmstadt, Germany) for 18 h at 37°C after which it centrifuged (at 240 rpm for 10 min), re-washed with sterile distilled water and standardized (Using MacCfarland standard). The inoculum was then added to the composite flour which was re-constituted in sterile distilled water in a transparent sterile container. The samples were left to ferment for 72 h. Daily (24 h) changes in pH and



**Figure 1.** Flow chart for the preparation of breadfruit-cowpea flour blends (Composites).

titratable acidity were determined. At the end of fermentation, the fermented samples were dried in air-oven and packaged in low density polyethylene pouches and stored at 8 °C prior to analyses. The fermentation process was carried out in three batches.

### Physico-chemical properties

#### *pH and titratable acidity*

The method described by AOAC (1998) was used to determine pH and titratable acidity of the fermenting medium. Samples were taken every 24 h during the fermentation period and homogenised according to the procedure described by Fayemi and Ojokoh (2012). Samples were measured using an Orion pH meter (Model 310, Orion Research Inc., Beverly, MA) equipped with glass electrode. The pH meter was calibrated with KOH buffer solutions of pH 7.0 and 4.0 before the measurements. The titratable acidity (TTA) was determined by titrating 20 ml of the homogenised sample against 0.1 M NaOH using phenolphthalein as an indicator. Values obtained were expressed as percent lactic acid. All analyses were carried out in triplicate.

#### *Proximate composition*

The moisture, crude protein (N × 6.25), crude fibre, crude fat and total ash contents of breadfruit-cowpea flour blends (composites) were determined before and after 72 h of fermentation using the method described by Association of Official Analytical Chemists'

(AOAC, 1998) approved methods 925.10, 920.87, 920.86, 920.39 and 923.03 respectively. Total carbohydrate content of the samples was calculated by difference method (subtracting the percent moisture, crude protein, crude fibre, crude fat, and ash from 100%)

### *Antinutritional contents*

Phytate and hydrogen cyanide (HCN) contents were determined by AOAC (1998) method. Oxalate content was measured according to the titrimetric method (AOAC, 1998).

### *Statistical analysis*

All measurements were carried out in triplicate. Analysis of variance (ANOVA) was conducted on the data at  $p < 0.05$  using MINITAB statistical software (Minitab® Release 14.13, Minitab Inc., USA). The least significant difference (LSD) at 95% confidence level was computed to ascertain where differences exist.

## RESULTS AND DISCUSSION

### *Proximate composition*

The proximate composition of fermented and unfermented breadfruit-cowpea flour blends is shown in

**Table 1.** Proximate composition of fermented and unfermented breadfruit-cowpea flour blends.

Proximate (%)	TvA		TvB		TvC		TvD		TvE		TvF	
	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented
Moisture	9.30 <sup>b</sup> ± 0.26	10.93 <sup>c</sup> ± 0.07	9.17 <sup>a</sup> ± 0.04	10.91 <sup>c</sup> ± 0.02	9.13 <sup>a</sup> ± 0.02	10.95 <sup>c</sup> ± 0.03	9.14 <sup>a</sup> ± 0.02	10.93 <sup>c</sup> ± 0.03	9.16 <sup>a</sup> ± 0.02	10.92 <sup>c</sup> ± 0.02	9.03 <sup>a</sup> ± 0.02	10.77 <sup>bc</sup> ± 0.02
Total ash	2.40 <sup>a</sup> ± 0.03	2.42 <sup>a</sup> ± 0.03	2.63 <sup>b</sup> ± 0.03	2.61 <sup>b</sup> ± 0.02	2.85 <sup>c</sup> ± 0.02	2.87 <sup>c</sup> ± 0.02	3.08 <sup>d</sup> ± 0.02	3.11 <sup>d</sup> ± 0.03	3.30 <sup>e</sup> ± 0.02	3.35 <sup>e</sup> ± 0.03	3.56 <sup>g</sup> ± 0.02	3.61 <sup>h</sup> ± 0.02
Crude fibre	3.25 <sup>h</sup> ± 0.02	2.88 <sup>f</sup> ± 0.02	2.94 <sup>g</sup> ± 0.03	2.68 <sup>e</sup> ± 0.03	2.69 <sup>e</sup> ± 0.02	2.45 <sup>d</sup> ± 0.03	2.47 <sup>d</sup> ± 0.02	2.24 <sup>c</sup> ± 0.02	2.26 <sup>c</sup> ± 0.03	2.88 <sup>f</sup> ± 0.02	2.06 <sup>b</sup> ± 0.02	1.83 <sup>a</sup> ± 0.03
Crude protein	4.00 <sup>a</sup> ± 0.20	7.25 <sup>c</sup> ± 0.15	5.86 <sup>b</sup> ± 0.04	11.23 <sup>i</sup> ± 0.16	7.87 <sup>d</sup> ± 0.02	13.35 <sup>h</sup> ± 1.15	9.93 <sup>e</sup> ± 0.04	15.21 <sup>i</sup> ± 0.03	11.91 <sup>g</sup> ± 0.05	17.24 <sup>k</sup> ± 0.02	14.18 <sup>j</sup> ± 0.06	24.14 <sup>l</sup> ± 0.02
Fat	2.27 <sup>a</sup> ± 0.25	3.59 <sup>g</sup> ± 0.07	2.55 <sup>b</sup> ± 0.04	3.74 <sup>h</sup> ± 0.02	2.71 <sup>c</sup> ± 0.02	3.05 <sup>d</sup> ± 0.03	2.92 <sup>d</sup> ± 0.02	4.18 <sup>i</sup> ± 0.03	3.13 <sup>e</sup> ± 0.03	4.36 <sup>j</sup> ± 0.02	3.34 <sup>f</sup> ± 0.02	4.72 <sup>k</sup> ± 0.03
Carbohydrate	80.14 <sup>g</sup> ± 1.43	73.58 <sup>e</sup> ± 0.47	77.09 <sup>f</sup> ± 0.11	68.77 <sup>d</sup> ± 0.17	74.72 <sup>e</sup> ± 0.03	66.66 <sup>d</sup> ± 0.02	72.46 <sup>e</sup> ± 0.04	64.41 <sup>bc</sup> ± 0.04	70.20 <sup>dc</sup> ± 0.02	63.18 <sup>b</sup> ± 0.02	67.92 <sup>d</sup> ± 0.02	55.13 <sup>a</sup> ± 0.13

Values are means ± standard deviation of triplicates. Values in the same row with different superscripts are significantly different at  $p < 0.05$ . TvA = 100:0 breadfruit flour to cowpea flour blend; TvB = 90:10 breadfruit flour to cowpea flour blend; TvC = 80:20 breadfruit flour to cowpea flour blend; TvD = 70:30 breadfruit flour to cowpea flour blend; TvE = 60:40 breadfruit flour to cowpea flour blend; TvF = 50:50 breadfruit flour to cowpea flour blend.

Table 1. Moisture content of the unfermented and fermented breadfruit-cowpea flour blends ranged from 9.03 - 9.30% and 10.77 - 10.95% respectively (Table 1). Apart from the unfermented breadfruit-cowpea flour blend (100:0) (TvA), the moisture contents for other flour blends were not significantly different ( $p > 0.05$ ) from each other (Table 1). Besides, the moisture contents for the fermented breadfruit-cowpea flour blends were not significantly different ( $p > 0.05$ ) from each other. However, moisture contents were significantly higher ( $p < 0.05$ ) for all the fermented samples compared with unfermented samples (Table 1). This result is in agreement with our previous study, where the breadfruit-cowpea flour blends were subjected to spontaneous fermentation (Ojokoh et al., 2013). Ojokoh et al. (2013) reported mean moisture content values of 9.09 and 10.52% for all unfermented and fermented samples. Moisture content of 9.61% has been reported for breadfruit flour (Ajani et al., 2012). Ariahu et al. (1999) also reported moisture content values of 9.8 and 10.4% for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed

food formulations respectively. Moisture content of flour provides an indication of shelf stability of the flour. It is however, depended upon the temperature and duration of the drying process. The low moisture contents recorded for all the flour blends suggest good keeping quality of the samples.

The total ash contents for unfermented and fermented breadfruit-cowpea flour blends ranged from 2.40 - 3.56% and 2.42 - 3.61% respectively (Table 1). These values increased significantly ( $p < 0.05$ ) as the amount of cowpea flour proportions in the breadfruit-cowpea flour blends increased for both unfermented and fermented samples. The total ash content values for the unfermented samples were not significantly different ( $p > 0.05$ ) from fermented samples, except for 60:40 and 50:50 breadfruit-cowpea flour blend samples which significantly increased ( $p < 0.05$ ) with fermentation (Table 1). The ash content values observed in this study were similar to the mean ash contents of 2.96 and 2.97% reported earlier for unfermented and fermented breadfruit-cowpea blend flours respectively (Ojokoh et al., 2013). Ash content of 1.05% has been reported for

breadfruit flour (Ajani et al., 2012). Values of 2.14, 2.82 and 1.27% have been reported for parboiled, boiled and fermented African breadfruit flours respectively (Onweluzo and Nnamuchi, 2009). Also, values of 1.8 and 2.5% ash have been reported for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed food formulations respectively (Ariahu et al., 1999). The increases in ash contents for both unfermented and fermented breadfruit-cowpea flour blends due to increase in cowpea flour proportion, could be attributed to the high ash content of cowpea. Ash content of 3.50% has been reported for cowpea flour (Darfour et al., 2012). Giami (1993) also reported ash content of 3.1% for cowpea flour. The results obtained from the measurements to determine ash content demonstrate that breadfruit-cowpea flour blends (fermented and unfermented) with 50:50 ratio (TvF) is richer in minerals when compared to the other formulations.

Crude fibre contents ranged from 2.06 - 3.25% for unfermented and 1.83 - 2.88% for fermented breadfruit-cowpea flour blends (Table 1). Increasing the proportions of cowpea significantly



decreased ( $p < 0.05$ ) the crude fibre contents of the breadfruit-cowpea flour blends. Similarly, fermentation caused a significant decrease ( $p < 0.05$ ) in the crude fibre contents of the breadfruit-cowpea flour blends. Mean fibre contents of 2.60 and 2.46% for unfermented and fermented breadfruit-cowpea flour blends were stated in our previous study (Ojokoh et al., 2013). High fibre content values of 3.9 and 4.5% have also been reported for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed food formulations respectively (Ariahu et al., 1999). Ajani et al. (2012) also reported fibre content of 1.26% for breadfruit flour. The decrease in crude fibre content of breadfruit-cowpea flour blends with increase in cowpea levels might be due to low content of fibre in cowpea. Giami (1993) reported crude fibre content of 2.1% for cowpea flour. Furthermore, the reduction in crude fibre content in the fermented breadfruit-cowpea flour blends could be attributed to enzymatic breakdown of fibre during fermentation by the *Lactobacillus plantarum* (Ojokoh et al., 2013). However, increased crude fibre content has been reported in fermented maize flour (Amankwah et al., 2009).

Crude protein contents ranged from 4.00 - 14.18 % for unfermented and 7.25 - 24.14% for fermented breadfruit-cowpea flour blends (Table 1). Increasing the proportions of cowpea significantly increased ( $p < 0.05$ ) the crude protein contents of the breadfruit-cowpea flour blends. Also, crude protein significantly increased ( $p < 0.05$ ) with fermentation of the breadfruit-cowpea flour blends (Table 1). This result compared favourably with our previous values, where mean protein content of 8.91 and 10.27% were recorded for unfermented and fermented breadfruit-cowpea blend flours respectively (Ojokoh et al., 2013). Ariahu et al. (1999) reported crude protein content of 16.4 and 16.8% for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed food formulations respectively. However, protein content of 1.34% has also been reported for breadfruit flour (Ajani et al., 2012). Protein contents (dry weight basis) of 18.65, 24.18 and 20.78% have been reported for parboiled, boiled and fermented African breadfruit flours respectively (Onweluzo and Nnamuchi, 2009). Other researchers also reported similar or greater increases in crude protein of cowpea (Giami, 1993) and various fermented Nigerian legumes and oilseeds (Akpapunam and Achinewhu (1985). The increased crude protein content of breadfruit-cowpea flour blends observed with increasing cowpea levels could be attributed to the high content of protein in cowpea (Giami, 1993). Also, the increases in crude protein content recorded with fermentation could be due to the activities and increase in number of *Lactobacillus plantarum* (Ojokoh et al., 2013). Amankwah et al. (2009) reported increased protein content in fermented maize flour. This increase in protein content was attributed to proteolytic activities of enzymes produced by microorganisms during

fermentation (Amankwah et al., 2009).

Fat contents of breadfruit-cowpea flour blends ranged from 2.27 - 3.34% and 3.59 - 4.72% for unfermented and fermented samples respectively (Table 1). Fat contents increased significantly ( $p < 0.05$ ) with increasing cowpea proportions of the breadfruit-cowpea flour blends. Fermentation also, significantly increased ( $p < 0.05$ ) the fat contents of the breadfruit-cowpea flour blends (Table 1). Mean fat contents of 2.82 and 3.28% were previously reported for unfermented and fermented breadfruit-cowpea blend flours respectively (Ojokoh et al., 2013). Fat content of 0.595% has also been reported for breadfruit flour (Ajani et al., 2012). Other researchers also reported increases in fat content (crude ether extract) for fermented African breadfruit flour (Onweluzo and Nnamuchi, 2009), fermented maize flour (Amankwah et al., 2009), fermented cowpea (Akpapunam and Achinewhu, 1985) and fermented fluted pumpkin seeds (Achinewhu and Isichei, 1990). The increased fat content of breadfruit-cowpea flour blends observed with increasing cowpea levels could be attributed to the high content of fat in cowpea. Also, the increases in fat content observed with fermentation could be due to the activities of lipolytic enzymes produced by *Lactobacillus plantarum* during fermentation (Onweluzo and Nnamuchi, 2009; Akpapunam and Achinewhu, 1985; Achinewhu and Isichei and Ojokoh et al., 2013).

Carbohydrate contents for unfermented and fermented breadfruit-cowpea flour blends ranged from 67.92 - 80.14% and 55.13 - 73.58% respectively (Table 1). These values decreased significantly ( $p < 0.05$ ) with increase in cowpea flour proportion in the breadfruit-cowpea flour blends for both unfermented and fermented samples. Fermentation was also found to significantly decreased ( $p < 0.05$ ) the carbohydrate contents of breadfruit-cowpea flour blends (Table 1). The carbohydrate content values observed in this study were similar to the mean carbohydrate contents of 73.58% and 70.32% reported earlier for unfermented and fermented breadfruit-cowpea blend flours respectively (Ojokoh et al., 2013). Carbohydrate content of 96.91% has been reported for breadfruit flour (Ajani et al., 2012). Values of 62.6 and 61.2% carbohydrate contents have also been reported for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed food formulations respectively (Ariahu et al., 1999). Other researchers also reported reduction in carbohydrate contents of cowpea (Giami, 1993) and various fermented Nigerian legumes and oilseeds (Akpapunam and Achinewhu (1985). The decreased carbohydrate contents recorded for both unfermented and fermented breadfruit-cowpea flour blends due to increase in cowpea flour proportion, could be attributed to the low carbohydrate content of cowpea compared to breadfruit. Furthermore, the reduction in carbohydrate content with fermentation could be due to utilization of fermentable sugars by *Lactobacillus plantarum* for growth and other metabolic

**Table 2.** Antinutritional contents of fermented and unfermented breadfruit-cowpea flour blends.

Content (mg/100 g)	TvA		TvB		TvC		TvD		TvE		TvF	
	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented	Unfermented	Fermented
Oxalate	2.78 <sup>b</sup> ± 0.02	0.90 <sup>a</sup> ± 0.02	2.76 <sup>b</sup> ± 0.03	0.81 <sup>a</sup> ± 0.02	2.70 <sup>b</sup> ± 0.02	0.78 <sup>a</sup> ± 0.02	2.63 <sup>b</sup> ± 0.03	0.75 <sup>a</sup> ± 0.01	2.59 <sup>b</sup> ± 0.02	0.63 <sup>a</sup> ± 0.03	2.36 <sup>b</sup> ± 0.02	0.38 <sup>a</sup> ± 0.02
Phytate	0.91 <sup>f</sup> ± 0.02	0.58 <sup>d</sup> ± 0.02	0.89 <sup>f</sup> ± 0.03	0.56 <sup>cd</sup> ± 0.02	0.84 <sup>f</sup> ± 0.02	0.42 <sup>bc</sup> ± 0.02	0.78 <sup>ef</sup> ± 0.02	0.38 <sup>ab</sup> ± 0.02	0.70 <sup>de</sup> ± 0.02	0.28 <sup>ab</sup> ± 0.02	0.68 <sup>de</sup> ± 0.02	0.24 <sup>a</sup> ± 0.02
HCN	2.73 <sup>c</sup> ± 0.03	0.02 <sup>a</sup> ± 0.003	2.55 <sup>bc</sup> ± 0.030	0.009 <sup>a</sup> ± 0.002	2.48 <sup>bc</sup> ± 0.02	0.006 <sup>a</sup> ± 0.002	2.38 <sup>bc</sup> ± 0.02	0.006 <sup>a</sup> ± 0.001	2.37 <sup>bc</sup> ± 0.02	0.004 <sup>a</sup> ± 0.001	2.28 <sup>b</sup> ± 0.020	0.004 <sup>a</sup> ± 0.001

Values are means ± standard deviation of triplicates. Values in the same row with different superscripts are significantly different at  $p < 0.05$ . TvA = 100:0 breadfruit flour to cowpea flour blend; TvB = 90:10 breadfruit flour to cowpea flour blend; TvC = 80:20 breadfruit flour to cowpea flour blend; TvD = 70:30 breadfruit flour to cowpea flour blend; TvE = 60:40 breadfruit flour to cowpea flour blend; TvF = 50:50 breadfruit flour to cowpea flour blend. HCN = Hydrogen cyanide.

activities (Ojokoh et al., 2013).

#### Antinutritional contents

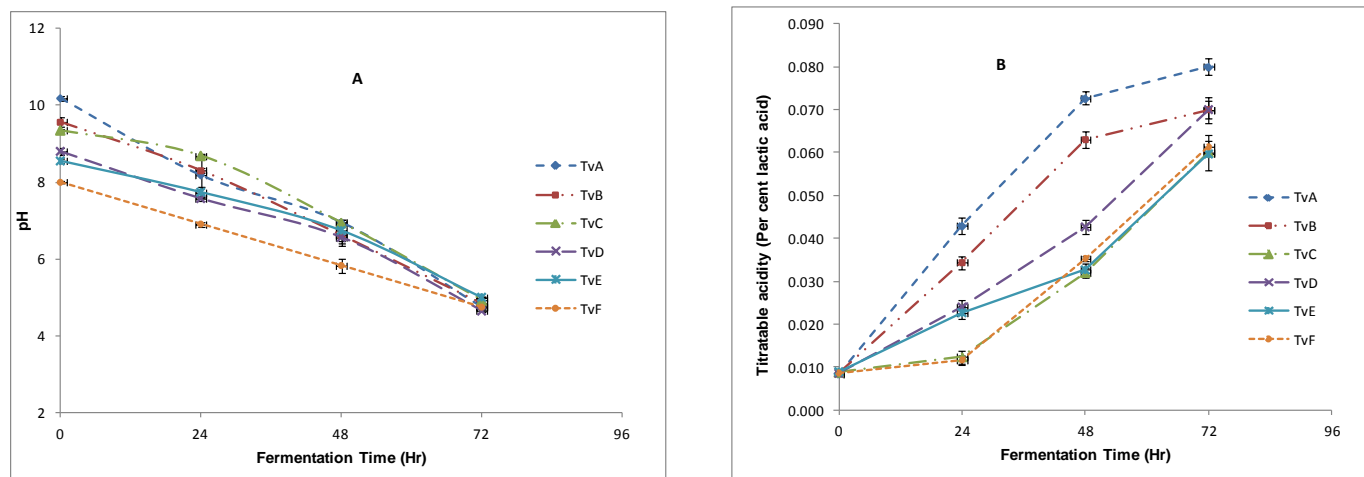
Oxalate contents for unfermented and fermented breadfruit-cowpea flour blends ranged from 2.36 - 2.78 mg/100 g and 0.38 - 0.90 mg/100 g respectively (Table 2). Though, the oxalate content decreased with increase in cowpea flour proportion in the breadfruit-cowpea flour blends, the changes were not significantly ( $p > 0.05$ ) different for both unfermented and fermented samples. However, fermentation significantly decreased ( $p < 0.05$ ) the oxalate contents of breadfruit-cowpea flour blends (Table 2). The oxalate contents observed in this study for unfermented breadfruit-cowpea blend flours were similar to values reported in our previous study, which ranged from 2.38 - 2.80 mg/100 g (Ojokoh et al., 2013). However, values for fermented breadfruit-cowpea blend flours were low compared to our previous results of 0.83 - 2.10 mg/100 g. The decreased oxalate contents for both unfermented and fermented breadfruit-cowpea flour blends due to increase in cowpea flour proportion, could be attributed to the low oxalate content of cowpea compared to breadfruit.

Also, the reduction in oxalate content with fermentation could be due to the activities of *Lactobacillus plantarum* (Ojokoh et al., 2013).

Phytate contents ranged from 0.59 - 0.93 mg/100 g for unfermented and 0.24 - 0.58 mg/100 g for fermented breadfruit-cowpea flour blends respectively (Table 2). Phytate contents for unfermented TvA, TvB, TvC and TvD were not significantly ( $p > 0.05$ ) different from each other (Table 2). However, values for unfermented TvE and TVF were significantly ( $p < 0.05$ ) different from unfermented TvA, TvB, TvC and TvD. Fermentation significantly decreased ( $p < 0.05$ ) the phytate contents of breadfruit-cowpea flour blends (Table 2). High phytic acid (phytate) contents of 1.76 g/kg and 1.17 g/kg have been reported for nongerminated-nonfermented soy-breadfruit seed and nongerminated-fermented soy-breadfruit seed food formulations respectively (Ariahu et al., 1999). Also, high phytate contents of 143.3 mg/100 g, 125 mg/100 g and 80.13 mg/100 g have been reported for parboiled, boiled and fermented African breadfruit flour (Onweluzo and Nnamuchi, 2009). The phytate contents recorded in the present study for unfermented breadfruit-cowpea blend flours were similar to values reported in our previous study, which ranged from 0.61 - 0.91 mg/100 g (Ojokoh et al.,

2013). Ojokoh et al. (2013) also reported reduction in phytate content of breadfruit-cowpea blend flours when spontaneously fermented. The reduction in phytate content with fermentation could partly be due to the activity of *Lactobacillus plantarum* (Ojokoh et al., 2013). Some microflora has been reported to possess phytase which breakdown phytate (Ojokoh, 2005).

Hydrogen cyanide contents for unfermented and fermented breadfruit-cowpea flour blends ranged from 2.28 - 2.73 mg/100 g and 0.004 - 0.020 mg/100 g respectively (Table 2). Hydrogen cyanide contents for unfermented and fermented breadfruit-cowpea flour blends reduced insignificantly ( $p > 0.05$ ) with increase in cowpea proportions (Table 2). However, fermentation significantly decreased ( $p < 0.05$ ) the hydrogen cyanide contents of breadfruit-cowpea flour blends (Table 2). Cyanide contents of 0.03 mg/100 g, 0.02 mg/100 g and 0.02 mg/100 g have been reported for parboiled, boiled and fermented African breadfruit flour (Onweluzo and Nnamuchi, 2009). The hydrogen cyanide contents recorded were similar to values of 2.28 - 2.65 mg/100 g and 0.006 - 0.01 mg/100 g reported for unfermented and fermented breadfruit-cowpea blend flours in our previous study (Ojokoh et al., 2013). The reduction in hydrogen cyanide content with



**Figure 2.** Changes in pH (A) and titratable acidity (B) of breadfruit-cowpea flour blends during fermentation period. TvA = 100:0 breadfruit flour to cowpea flour blend; TvB = 90:10 breadfruit flour to cowpea flour blend; TvC = 80:20 breadfruit flour to cowpea flour blend; TvD = 70:30 breadfruit flour to cowpea flour blend; TvE = 60:40 breadfruit flour to cowpea flour blend; TvF = 50:50 breadfruit flour to cowpea flour blend. Error bars =  $\pm$  standard deviations.

fermentation could be attributed to enzyme activities of *Lactobacillus plantarum* (Kobawila et al., 2005).

The reduction in antinutritional contents of the breadfruit-cowpea flour blends after fermentation suggests bioavailability of the rich nutrients present in the fermented products.

### Physico-chemical properties

pH for unfermented breadfruit-cowpea flour blends ranged from 7.99 - 10.18 (Figure 2A). Increasing the cowpea flour proportion of the breadfruit-cowpea flour blends significantly decreased ( $p < 0.05$ ) the pH of the samples. The titratable acidity (TTA) (expressed as % lactic acid) for unfermented breadfruit-cowpea flour blends ranged from 0.008 - 0.009 (Figure 2B). Increasing the cowpea flour content of the breadfruit-cowpea flour blends however did not significantly ( $p > 0.05$ ) change the TTA of the samples. The pH values of the breadfruit-cowpea flour blends decreased significantly ( $p < 0.05$ ) with increase in fermentation period (Figure 2A). Also, TTA of the breadfruit-cowpea flour blends significantly ( $p < 0.05$ ) increased with increase in fermentation period (Figure 2B). Similar decrease in pH and increase in TTA due to spontaneous fermentation have been reported for breadfruit-cowpea blend flours (Ojokoh et al., 2013). Ariahu et al. (1999) also reported similar decrease in pH and increase in TTA during fermentation of nongerminated and germinated soy-breadfruit seed food formulations. Amankwah et al. (2009) reported significant decrease in pH and increased titratable acidity in fermented maize flour. The reduction in pH and increase in TTA during fermentation could be attributed to the production of lactic acid by *Lactobacillus plantarum*

(Ariahu et al., 1999; Ojokoh et al., 2013). Lactic acid bacteria have been reported to degrade carbohydrates (Table 1) resulting in acidification (Ariahu et al., 1999; Ojokoh et al., 2013). High acidity in fermented legumes has been reported to reduce the incidence of diarrhea among infants (Mensah et al., 1990).

### Conclusions

The results from this study show that fermentation of breadfruit-cowpea flour blends using pure strains of *Lactobacillus plantarum* (FO-12) improves the nutritional qualities of the blends. This implies that fermented breadfruit-cowpea flour blends have potentials in the formulation of weaning foods for the management of Protein-Energy Malnutrition (PEM).

### Conflict of interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

## Comparison of diagnostic methods for bacterial vaginosis

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**Bacterial vaginosis is a disease affecting many women all over the world, being asymptomatic or symptomatic and producing obstetric and gynaecological complications. The aim of this study was to compare three methods for the diagnosis of bacterial vaginosis. For this cross-sectional descriptive study, vaginal swabs from 101 women attending Máximo Gómez Clinic in Holguín, Cuba, from April to May 2011 were used. One clinical and two laboratory methods were applied. The specimens were processed according to the standard ethical and laboratory protocols. Kappa index was used to determine the concordance between two methods at once, and categories from three methods were compared and analysed. A total of 37 (36.6%), 29 (28.7%) and 42 (41.6%) patients were diagnosed with bacterial vaginosis according to Amsel's, Nugent's and Claeys' methods, respectively. Kappa index between methods was 0.6872 (Amsel-Nugent), 0.7522 (Nugent-Claeys) and 0.8134 (Amsel-Claeys). As predictor of bacterial vaginosis, from Amsel's criteria, homogeneous white-greyish discharge was the least specific and whiff test was the most specific, while whiff test was the least sensitive and pH exceeding 4.5 was the most sensitive. The diagnosis of bacterial vaginosis by the three methods shows moderate concordance, being Claeys' method the most effective. Nugent's method seems to make a sub-diagnosis of the disease and Amsel's method contains criteria with poor sensitivity and specificity.**

**Key words:** Bacterial vaginosis, polybacterial disease, kappa index, diagnosis, Amsel's method, Nugent's method, Claeys' method.

### INTRODUCTION

Bacterial vaginosis (BV) is one of the most frequent female reproductive tract diseases with highest prevalence (20% to 40%) in underdeveloped countries and in population with risk factors such as promiscuity (De Backer et al., 2007;

Verstraelen et al., 2010). It has been accepted for several years the polybacterial origin of the disease, where the proliferation of Gram negative or Gram variable strict anaerobes or facultative bacteria, that can be found in

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gastrointestinal tract and in few concentration in healthy vaginal ecosystem, can be associated with the diminishing or total depletion of vaginal lactobacilli (Marrazzo, 2006).

In healthy vagina of fertile women, 4 species of lactobacilli are predominant: *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* (Antonio et al., 1999; Vázquez et al., 2002), each one with different morphology, Gram staining features and protective effect in the vaginal ecosystem. On the other hand, the qualitative and quantitative composition of BV is variable, complex and not completely understood and may include more than 80 different genera and thousands of species such as *G. vaginalis*, *Atopobium vaginae*, *Bacteroides*, *Prevotella*, *Porphyromonas*, *Peptostreptococcus*, *Mobiluncus*, *Mycoplasma*, *Corynebacterium*, *Enterococcus*, *Bifidobacterium*, *Megasphaera*, *Leptotrichia*, *Eggerthella*, *Dialister*, *Clostridium*, *Gamella*, *Veillonella*, *Fusobacterium*, *Acetivibrio*, *Eubacterium*, *Aerococcus*, *Olsenella*, etc. (Forsum et al., 2005; Oakley et al., 2008; Srinivasan and Fredricks, 2008; Zongxin et al., 2010).

The disease may follow an asymptomatic course, affecting diagnosis and treatment with important consequences from the epidemiological point of view. Symptomatic patients may have vaginal discharge and vulvar burning, increasing the risk of acquisition of sexual transmitted diseases and acquired immunodeficiency syndrome, and can cause also major obstetric and gynecological complications, threatening the life of the mother and fetus (Watts et al., 1990; Rado et al., 2001; Lim et al., 2010; Shilpa et al., 2012).

Diagnosis of BV has been a controversial issue. Although some clinical and microbiological diagnostic methods are available, a powerful method does not exist. In primary care and routine diagnosis Amsel's method is used, based mainly on clinical evidence and characteristics of vaginal discharge (Amsel et al., 1983), whereas Nugent's method is considered the standard method and is used in epidemiological and research studies, based on the amount of different bacterial morphotypes present in the vaginal ecosystem (Nugent et al., 1991).

Claeys' method (also known as Ison/Hay), created in 2002 by Ison and Hay and modified in 2005 by Verhelst et al, based on the rate of lactobacilli and BV-associated bacterial morphotypes, is one of the methods proposed in the past 20 years that stands out for its success in the characterization of vaginal microbiota and its ability to detect those patients at increased risk of developing the disease.

Several attempts to improve diagnosis have been developed, ranging from modifications of the Amsel's method, by using only some of the criteria proposed by the author, to serological tests for antigen detection of certain bacteria (such as *G. vaginalis*), or molecular biology approaches for detection of nucleic acids by hybridization or polymerase chain reaction (PCR) (Ramírez et al., 2004;

Vázquez et al., 2004; López et al., 2008; Menard et al., 2010; Cartwright et al., 2012). All these procedures attempt to identify one or only some of the bacteria involved in BV that could even be found in the vagina of healthy patients, therefore they have low specificity, lacking real value to diagnosis.

The present study was planned to compare the diagnosis of BV according to three different methods (Amsel, Nugent and Claeys), using kappa index and analysing the behaviour of the different categories among methods.

## METHODOLOGY

### Study design

For this cross-sectional descriptive study with accidental sampling, vaginal swabs from 101 post-menarchical women attending to Máximo Gómez Clinic, in Holguín, Cuba, from April to May 2011 were used. The following exclusion criteria were applied: pregnant patients, patients undergoing antibiotic treatment 72 h before sampling, patients with any vaginal bleeding and patients who had unprotected sex the day before sampling.

### Ethical consideration

All ethical considerations and legal regulations existing in Cuba were taken into account to ensure the principle of voluntariness and anonymity of the patients in this study as well as knowledge of the objectives thereof and consent to the publication of results.

### Procedures

The specimens were processed according to the standard ethical and laboratory protocols. One clinical (Amsel) and two laboratory (Nugent and Claeys) methods were used. A non-lubricated speculum was inserted into the vagina of the patient and two swabs were taken, one was used for Gram stain and the other for the evaluation of the Amsel's criteria (vaginal pH, whiff test, clue cells). Each method was carried out by a separate examiner. The procedure for each of the methods was made as described below.

### ***BV diagnosis by Amsel's method (Amsel et al., 1983)***

According to Amsel's method a patient is positive for BV when 3 of the following 4 criteria are present:

- Vaginal pH above 4.5.
- A thin, homogeneous ("milk-like consistency") white-greyish vaginal discharge, independently of quantity.
- Accentuation of the fishy odor of the vaginal discharge with the addition of 10% potassium hydroxide (whiff test).
- Clue cells on microscopic examination of vaginal swabbing samples in saline.

### ***BV diagnosis by Nugent's method (Nugent et al., 1991)***

Nugent's method is based on counting bacterial morphotypes present on Gram stain of vaginal discharge and morphotypes are classified into three groups:

**Table 1.** Individual score by morphotype according to 0/+4 grading.

Score	Lact. morphotype	G-B. morphotypes	M. morphotypes
0	4+	0	0
1	3+	1+	1+ 2+
2	2+	2+	3+ 4+
3	1+	3+	
4	0	4+	

Total score interpretation and BV diagnosis by Nugent's method: 0-3 Negative for BV, 4-6 IMB (Intermediate microbiota: negative for BV), 7-10 Positive for BV.

(a) *Lactobacillus* morphotypes (Lact.): Gram positive rods.

(b) *Gardnerella-Bacteroides* morphotypes (G-B): Gram negative or Gram variable coccobacilli.

(c) *Mobiluncus* morphotypes (M): Gram negative or Gram variable, weakly stained and sharp ended curved rods.

Morphotypes are scored as the average number seen per oil immersion field (5 to 10 fields) and the following values are assigned:

0 → average = 0  
 1+ → 0 < average ≤ 1  
 2+ → 1 < average ≤ 4  
 3+ → 4 < average ≤ 30  
 4+ → average > 30

The values obtained according to the previous grading (0 to 4+) are scored as shown in Table 1. Total score is obtained with the sum of partial scores from each bacterial morphotype.

#### **BV diagnosis by Claeys' method (Ison and Hay, 2002; Verhelst et al., 2005)**

Claeys' method determines the relative concentration of Gram positive rods (lactobacilli) and BV-associated bacterial morphotypes without counting the bacterial morphotypes but determining the rate. Vaginal ecosystem can be graded into 8 different categories as follow:

**Grade 0:** vaginal epithelial cells with very little or no bacterial morphotypes found.

**Grade I:** when *Lactobacillus* morphotypes are present only; 4 different types can be found according to their morphology and staining features:

**Grade Ia:** when only *Lactobacillus crispatus* cell types (plump, mostly short rods, strongly stained) are present.

**Grade Ib:** when only other *Lactobacillus* cell types are present (smaller or more elongated and less stained than in Ia smears).

**Grade Iab:** when both *L. crispatus* and other lactobacilli are present.

**Grade I-like:** when other species of *Lactobacillus* and *Bifidobacterium* (Gram positive rods, either quite small or short or otherwise irregularly shaped with clubbing, curved edges and irregular staining and often arranged like Chinese letters or diptheroid cell types) are present.

**Grade II:** reduced *Lactobacillus* morphotypes with mixed BV-associated bacterial morphotypes.

**Grade III:** predominantly BV-associated bacterial morphotypes (Gram negative or Gram variable coccobacilli and Gram variable, weakly stained and sharp ended curved rods) are present.

**Grade IV:** epithelial cells covered predominantly or only with Gram positive cocci.

Final diagnosis of BV can be made as follow:

**Grades 0, I y IV** are considered negative for BV.

**Grade II** is considered as intermediate microbiota (IMB), but still negative.

**Grade III** is considered positive for BV.

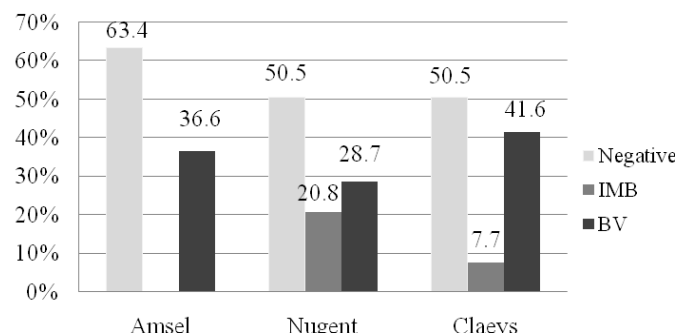
#### **Statistical analysis**

Kappa index was used to determine the concordance between two methods at once (weighted if necessary) as well as 95% confidence intervals (CIs) and minimum and maximum kappa value, calculated with EPIDAT program for windows, version 3.1. In the case of Claeys-Nugent pair, kappa weighted manually (Appendix 1) was taken as the most successful, because it gives more importance to the differences between positive and negative diagnoses that between subcategories within the same negative diagnosis. Kappa values near from 0 indicate low agreement whereas values near from 1 indicate high agreement (Lantz and Nebenzahl, 1996; Abaira, 2000).

## **RESULTS**

### **Diagnosis of BV using three different methods**

According to Amsel's, Nugent's and Claeys' methods BV was found in 36.6%, 28.7% and 41.6% of patients, respectively (Graphic 1). Intermediate microbiota (IMB) category differs considerably between Nugent's and Claeys' methods.



**Graphic 1.** Diagnosis of BV according to Amsel's, Nugent's and Claeys' methods. Amsel's method contains only two categories (Negative and BV). IMB: Intermediate microbiota.

**Table 2.** Kappa index between Nugent's and Claeys' methods.

Nugent	Claeys		
	Negative	IMB	BV
Negative	48	3	0
IMB	3	5	13
BV	0	0	29

Kappa: 0.7522 (manual weighted); CI: 0.6549-0.8495 IBM: Intermediate microbiota, BV: bacterial vaginosis.

**Table 3.** Kappa index between Amsel's and Nugent's methods.

Nugent	Amsel	
	Negative	BV
Negative	61	11
BV	3	26

Kappa: 0.6872; CI: 0.5382-0.8361; Minimum Kappa: - 0.0745; Maximum Kappa: 0.7280 BV: bacterial vaginosis.

**Table 4.** Kappa index between Amsel's and Claeys' methods.

Claey	Amsel	
	Negative	BV
Negative	57	2
BV	7	35

Kappa: 0.8134; CI: 0.6977-0.9290; Minimum Kappa: - 0.0466; Maximum Kappa: 0.8232 BV: bacterial vaginosis.

## Kappa index

Tables 2, 3 and 4 show kappa indexes between pairs of methods Nugent-Claeys (0.7522), Nugent-Amsel (0.6872) and Claeys-Amsel (0.8134). For Nugent-Claeys kappa index manual weighted was used (Appendix 1).

Nineteen patients did not coincide on the diagnosis of BV by Nugent's and Claeys' methods (Table 2), 14 patients did not coincide on the diagnosis by Nugent's and Amsel's methods (Table 3), whereas 9 patients did not coincide on the diagnosis by Claeys' and Amsel's methods (Table 4).

## Analysis of coincidence between the categories of the three used methods

Homogeneous white-greyish vaginal discharge was

observed in 74 patients, pH above 4.5 in 51 patients, positive whiff test in 28 patients and clue cells was found in 51 patients (Table 5). Analysing the behaviour of each of the Amsel's criteria in relation to the diagnosis made by the three methods was found that homogeneous white-greyish vaginal discharge was present in more than 68% of patients with and without BV.

From 23% to 33% of diagnosed patients without BV had pH above 4.5, while in patients diagnosed with BV was present in more than 88%. Whiff test was positive in only 8.3% of patients without BV by Nugent's method and in no patient by the other two methods, whereas it was observed from 66% to 76% in patients with BV. The presence of clue cells had a similar behaviour to pH (Table 5).

Graphic 2 shows the behaviour of three of the Amsel's criteria (excluding vaginal discharge) according to Nugent's subcategories. As most relevant results 15 patients (71.4%) presented pH exceeding 4.5 and six patients (28.6%) presented positive whiff test, both in intermediate microbiota category (group B) from Nugent's method. The six patients (28.6%) in group B with positive whiff test match with a score of 5 or 6 according to Nugent's method, and all contain an average of more than 30 morphotypes characteristic of BV and less than 8 *Lactobacillus* morphotypes per field (data no shown).

In the comparison of Amsel's and Claeys' categories (Graphic 3) 11 patients (23.4%) presented clue cells in negative category (Grade I) and 4 patients (100%) presented pH exceeding 4.5 in negative category (Grade 0 and IV). Moreover positive whiff tests were only found in BV positive category (Grade III), being completely absent in the other categories.

Of the 11 patients (23.4%) in Group D that were positive for clue cells (Graphic 3), 7 correspond with Grade Ia (presumably *L. crispatus*), and 4 correspond with Grade I-like (other *Lactobacillus* and *Bifidobacterium* species) (data not shown). Of the 4 patients (100%) in Group E with pH above 4.5, 2 showed no bacterial morphotypes, one showed less than 14 *Lactobacillus* morphotypes and one showed less than 4 BV-associated bacterial morphotypes, also three of them were post-menopausal women (data not shown).

In the comparison of Nugent's and Claeys' categories (Graphic 4) three patients classified as negative by Nugent (A) were classified as IMB (grade II) by Claeys, and all of them presented an average of more than 30 *Lactobacillus* morphotypes and a range between 5 and 23 BV-associated bacterial morphotypes per field (data not shown). Three patients classified as IMB (B) by Nugent were classified as negative (Grade 0 and IV) by Claeys (Graphic 4).

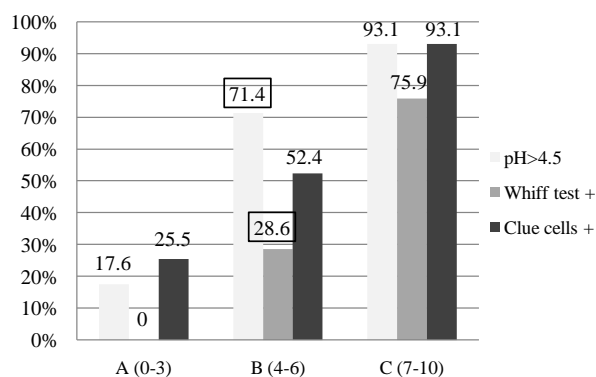
Thirteen patients classified as IMB (B) by Nugent were classified as positive for BV by Claeys (Grade III) (Graphic 4), and all of them with an average less than 9 *Lactobacillus* morphotypes and more than 25 BV-associated bacterial morphotypes per field (data not shown).



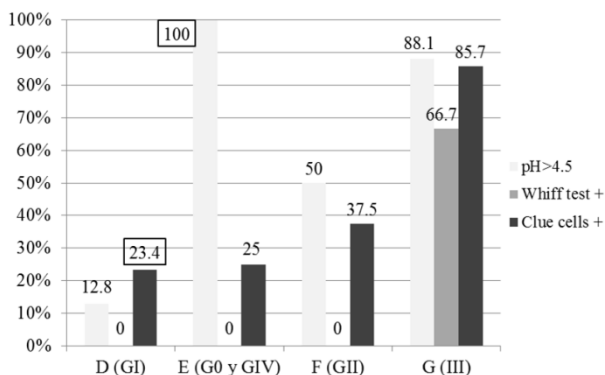
**Table 5.** Individual behaviour and sensitivity and specificity of Amsel's criteria according to the three methods used.

Method	Result	Discharge (74)		pH>4.5 (51)		+ Whiff test (28)		+ Clue cells (51)	
		No.	%	No.	%	No.	%	No.	%
Amsel	Negative	44	68.8 <sup>†</sup>	16	25.0	0	0	16	25.0
	BV	30	81.1 <sup>‡</sup>	35	94.6	28	75.7	35	94.6
Nugent	Negative	51	70.8	24	33.3	6	8.3	24	33.3
	BV	23	79.3	27	93.1	22	75.9	27	93.1
Claeys	Negative	41	69.5	14	23.7	0	0	15	25.4
	BV	33	78.6	37	88.1	28	66.7	36	85.7

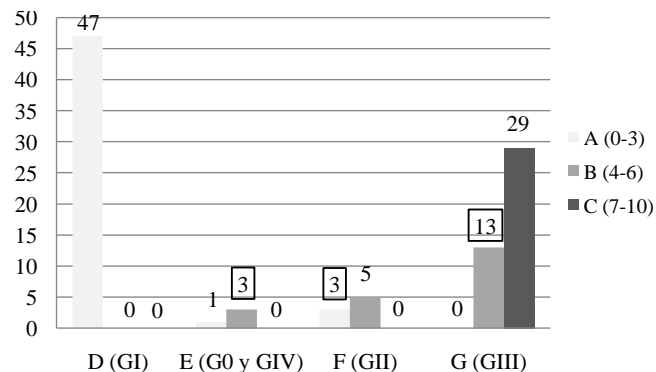
<sup>†</sup>This value agrees with "100 - specificity" of homogenous white or white-greyish vaginal discharge as a predictor of BV, the same goes for the rest of the Amsel's criteria for each method. <sup>‡</sup>This value agrees with the sensitivity of homogenous white or white-greyish vaginal discharge as a predictor of BV, the same goes for the rest of the Amsel's criteria for each method. BV: bacterial vaginosis.



**Graphic 2.** Behaviour of three of the Amsel's criteria according to the categories of Nugent's method. Nugent's categories: A (Negative with total scores from 0 to 3), B (Intermediated microbiota with total scores from 4 to 6), C (positive for BV with total score from 7 to 10).



**Graphic 3.** Behaviour of three of the Amsel's criteria according to the categories of Claeys' method. Claeys' categories: D (Negative with prevalence of *Lactobacillus* morphotypes), E (Negative with absence of bacterial morphotypes or prevalence of Gram positive cocci), F (Intermediated microbiota with similar rates of *Lactobacillus* morphotypes and BV-associated bacterial morphotypes), G (Positive for BV with prevalence of BV-associated bacterial morphotypes).



**Graphic 4.** Agreement between Nugent's and Claeys' categories. Nugent's categories: A (Negative with total scores from 0 to 3), B (Intermediated microbiota with total scores from 4 to 6), C (positive for BV with total score from 7 to 10). Claeys' categories: D (Negative with prevalence of *Lactobacillus* morphotypes), E (Negative with absence of bacterial morphotypes or prevalence of Gram positive cocci), F (Intermediated microbiota with similar rates of *Lactobacillus* morphotypes and BV-associated bacterial morphotypes), G (Positive for BV with prevalence of BV-associated bacterial morphotypes).

## DISCUSSION

The positive diagnosis of BV in most underdeveloped countries is above 20%, as reported in other studies (Ison and Hay, 2002; Tokyol et al., 2004; Demba et al., 2005; Arce-Espinoza, 2009) as well as in this study. The number of positive cases according to Amsel's method occupies an intermediate position compared with the other two methods, which are expected to have a better match between them (Nugent and Claeys) because both methods are based on the analysis of bacterial morphotypes. Amsel's method is dichotomous, and has only two categories (negative and positive) and the characterization of vaginal microbiota status is less accurate in comparison with Nugent and Claeys, having three categories

(intermediate microbiota is added).

Kappa index determines the degree of agreement between two or more measurements of the same variable, but is unable to determine superiority between them. Although the largest number of discrepancies were observed between Nugent and Claeys, kappa value was not lower because the disagreements occurred between consecutive subcategories and never between extremes subcategories.

The discrepancy in the diagnosis of positive cases between Nugent's and Claeys' methods might indicate an overestimation in the diagnosis of BV by Claeys' method or an underestimation by Nugent's method. Taking into consideration that Claeys' method determines the proportion of bacterial morphotypes presents; the previous discrepancy suggests that Nugent is able to detect mainly the most severe cases of BV. The same analysis can be done with the discrepancies between Amsel and Nugent; also previous studies have found that Amsel's method is more specific than Nugent's method (Kent, 1991; Livengood, 2009).

Amsel's and Claeys' methods show the lowest discrepancy. Ison and Hay found that according to Amsel, Claeys is more sensitive than specific (Ison and Hay, 2002). Although Amsel's method is widely accepted, the authors of this document consider that Claeys' method is able to characterize better the vaginal microbiota and further publications will show some improvements that can be done in Claeys' method.

A study by Forsum and colleagues in 2002 showed kappa values below 0.75 between Amsel and Nugent, 0.89 between Nugent and Claeys and 0.82 between Amsel Claeys. In this study kappa index between Nugent and Claeys is lower, and may be because of the use of different weights kappa.

The internal comparison of the categories of the three methods can yield important information about the main differences and weaknesses of methods. According to the behaviour of individual Amsel's criteria and its capacity of act as predictor of BV, homogeneous white-greyish vaginal discharge was the least specific and whiff test was the most specific, while whiff test was the least sensitive and pH exceeding 4.5 was the most sensitive. Coinciding with this study, several authors have found vaginal discharge as the most controversial of the Amsel's criteria (Hellberg et al., 2001; Livengood, 2009). Colour, quantity and consistency of vaginal discharge are very subjective characteristics that can vary depending on local illumination, visual acuity and personal opinion of the examiner, as well as individual patient characteristics: in addition, in cases of concomitant infections with BV (candidiasis or trichomoniasis) the characteristics of leucorrhoea change drastically. Ramirez and colleagues in 2004, found sensitivity and specificity for vaginal pH (relative to the Amsel's method) similar to this study (Ramírez et al., 2004). The presence of residues of semen

or blood in the vagina at the time of sampling can increase the vaginal pH and thus affect the specificity of this criterion (Livengood, 2009). The sensitivity of the whiff test as predictor of BV can be affected by the presence of bacteria with variable capacity to produce trimethylamine (volatile compound derived from the metabolism of amino acids). Ramirez et al. found in 2004 a slightly lower sensitivity and specificity than in this study. Other studies have also found higher values of sensitivity than specificity for clue cells (Hellberget al., 2001; Ramírez et al., 2004).

The absence of whiff test in negative patients in 2 of the methods (Amsel and Claeys), even in the IMB category, suggests a good specificity of the criterion, so Nugent's method seems to makes an undervaluation of the BV (detecting only severe cases). The above assertion could explain the high number of patients who belong to IMB category (according to Nugent) having many of the Amsel criteria.

The normal microbiota of the vagina changes during the biological development of women. Different microbial successions occur, depending primarily on changes in hormone levels, although other factors may influence such as sexual behaviour, health habits and social behaviour in general.

All these factors can affect qualitatively and quantitatively the vaginal microbiota. Lactobacilli may be absent or diminished in post-menopausal women, even in some fertile healthy women (Turovskiy et al., 2011), increasing considerably the vaginal pH, and Amsel's method does not assess this normal physiological difference in women of reproductive age and post-menopausal, affecting the specificity of the criterion. Something similar happens with Nugent's method, because it does not take into account this lactobacilli decrease or absence as a totally normal or physiological process.

The detection of clue cells on direct smear of vaginal secretion requires experience and properly calibrated microscopes. *Lactobacillus* morphotypes classified as GIa are more adherent to vaginal epithelial cells than other lactobacilli (McLean and Rosenstein, 2000), while those classified as GI-like are smaller and irregular (Verhelst et al., 2005) and in both cases may mislead the examiner with the presence of false clue cells.

The overall score for Nugent's method increases with the increasing of BV-associated morphotypes and/or with decreasing of *Lactobacillus* morphotypes. In some cases only with the absence of lactobacilli, this method can score a 4 (MBI), when in fact there is no evidence of BV (as occur with post-menopausal women), so Nugent's method should be modified or redesigned to achieve a more accurate diagnosis of BV (analysing the cutoff point).

## Conclusions

The diagnosis of BV by Amsel's, Nugent's and Claeys'

methods shows moderate concordance, being Claeys' method the most effective. Nugent's method seems to make a sub-diagnosis of the disease, due to a low cutoff point, and also overestimates the relevance of reduced lactobacilli in the diagnosis of the disease.

Amsel's method contains criteria with poor sensitivity and specificity, being vaginal discharge the least specific. The characteristics of the vaginal microbiota of post-menopausal women should be taken into consideration for an accurate diagnosis of the disease.

### Conflict of interests

The author(s) have not declared any conflict of interests.

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**Appendix 1.** Manual weighting values for kappa index between Nugent's and Claey's methods.

<b>Parameter</b>	<b>Neg.</b>	<b>IMB</b>	<b>BV</b>
<b>Neg.</b>	1.0	0.5	0.0
<b>IMB</b>	0.5	1.0	0.3
<b>BV</b>	0.0	0.3	1.0

Full Length Research Paper

## Effect of antagonistic Rhizobacteria coinoculated with *Mesorhizobium ciceris* on control of fusarium wilt in chickpea (*Cicer arietinum* L.)

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The antagonistic activity against *Fusarium oxysporum* f. sp. *ciceris* was determined for 40 chickpea rhizobacteria. Twenty eight isolates showed antagonistic activity against test fungus ranging from 18.2 to 41.8%. Characterization of the antagonistic attributes showed that all the antagonistic isolates produced diffusible and volatile antifungal metabolites in terms of growth inhibition, maximum being with the isolates 39P (77.8%) and 15B (64.2%), respectively. Nineteen of the isolates showed catechol and hydroxamate type siderophore production. All the isolates produced ammonia and twelve showed HCN production. On the basis of their antagonistic and PGP functionality traits, five isolates (2B, 7B, 28P, 34P and 38P) were selected for glass house studies on two chickpea varieties (JG-62 and GPF-2). Isolates 28P, 34P and 38P were found to be most promising for wilt control and plant growth promotion. Isolate 38P reduced the wilt incidence to 44.6% which was at par with fungicide treatment (55.5%) and had a significant edge over negative control (85%) in the chickpea variety JG-62. Similar trend of wilt incidence was observed in GPF-2 variety. Green house experiments on two varieties of chickpea JG-62 and GPF-2 showed that seed treatment with plant growth promoting rhizobacteria (PGPR) + *Mesorhizobia* had a synergistic effect in terms of disease control and growth promotion as compared to use of single bioinoculants, thus positively influencing plant microbe interaction.

**Key words:** Antagonism, chickpea, *Fusarium*, plant growth promoting rhizobacteria (PGPR), *Rhizobium*.

### INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is a major constraint to chickpea (*Cicer arietinum* L.) cultivation throughout the world and especially in Indian subcontinent where chickpea is a commonly grown pulse crop. Presently, it occupies an area of 8 million hectare and its production is 7.1 million tons (Nikamet al., 2011). Despite the high total production, yields of chickpea are low due to attack of different diseases including fusarial wilts which can cause up to 100% yield losses annually (Pandeet al., 2010).

Plant growth promoting rhizobacteria (PGPR) have been proved as biocontrol agents of soil borne plant pathogens, offer an attractive alternative to chemical fertilizers, pesticides and supplements. Thus, the use of PGPR is

steadily increasing in agriculture (Ashrafuzzaman et al., 2009). Plant growth promoting rhizobacteria are a heterogeneous group of bacteria that can be found in the rhizosphere at root surfaces and in association with roots which can improve the extent or quality of plant growth directly or indirectly (Joseph et al., 2007; Datta et al., 2011). The direct mechanism involves the N<sub>2</sub> fixation (Wani et al., 2007), solubilization of insoluble phosphorus (Khan et al., 2009), sequestering of iron by production of siderophore (Rajkumar et al., 2006), production of phytohormones such as, auxins, cytokinins and gibberellins (Godhino et al., 2010) and their transport to the developing plants or facilitating the uptake of nutrients from the recipient environment. The indirect mechanisms involve

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production of siderophores that chelate iron, making it unavailable to phytopathogens, antagonism by synthesis of volatile and diffusible antifungal metabolites such as phenazine and hydrogen cyanide, the ability to successfully compete with pathogens for nutrients and niches on the root to induce systemic resistance (Nelson, 2004; Saharan and Nehra, 2011).

Rhizobacteria are reported to play an important role in biocontrol via production of volatile antifungal compounds such as ammonia, aldehydes, alcohols, ketones and sulfides (El-Katatany et al., 2003). The production of diffusible antifungal metabolites with biocontrol activity is also reported. Phenazine is a diffusible potent green-pigmented antimicrobial metabolite implicated in antagonism (Tjeerdvan et al., 2004). A large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter* (Ahmad et al., 2008), *Bacillus* (Cakmakci et al., 2007; Ahmad et al., 2008), *Beijerinckia* (Thuler et al., 2003), *Burkholderia* (Govindarajan et al., 2006), *Klebsiella* (Govindarajan et al., 2007), *Serratia* (Gyaneshweret al., 2001) have been reported to enhance plant growth, reduce pathogenic growth and disease development in various crops.

The objective of the present study was to isolate rhizobacteria from the chickpea rhizosphere with potent antagonistic activity against *F. oxysporum* f. sp. *ciceris* and to determine the ability of the selected bacterial antagonists alone and in combination with *Mesorhizobium ciceris* to suppress fusarium wilt under green house conditions.

## MATERIALS AND METHODS

### Isolation of rhizobacteria

The rhizobacteria were isolated from twenty different soil samples collected from different chickpea growing fields in Punjab, Uttar Pradesh and Palampur. All the microbial strains were isolated on their respective media; *Bacillus* and *Serratia* spp. on Nutrient agar, *Pseudomonas* spp. on King's B agar.

### Characterization of rhizobacteria isolates

Selected bacterial isolates were characterized on the basis of Gram's reaction, catalase production, nitrate reduction, starch hydrolysis and methyl red test as per the standard methods (Cappuccino and Sherman, 1992).

### Screening for antagonistic rhizobacteria against *F. oxysporum* sp. *ciceris*

#### Antagonistic activity

Antagonistic activity of the 48 bacterial isolates against *F. oxysporum* f. sp. *ciceris* (Department of Plant Breeding and Genetics PAU, Ludhiana, India) was evaluated based on dual culture technique (Lemessa and Zeller, 2007) and replicated thrice. Radial growth of the test fungus was measured and percentage growth inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = (R - r)R \times 100$$

Where,  $r$  is the radius of the fungal colony opposite the bacterial colony and  $R$ , is the maximum radius of the fungal colony in the absence of the bacterial colony.

### Inhibition of fungal mycelium proliferation

One milliliter of 24 h old bacterial culture and a 5 mm disc of test fungus were inoculated in 50 ml of potato dextrose media in 250 ml conical flasks at 25°C on a rotary shaker (three replication per isolate). Broth inoculated only with fungus served as control. The differences in dry weights between the fungus and the bacterium or the control cultures were recorded by passing 48 h grown dual cultures through preweighed filter paper. The filter papers were dried for 24 h at 70°C and weighed. The percent reduction in weight of the test fungus was calculated using the formula:

$$\text{Reduction in weight (\%)} = (w_1 - w_2) / w_1 \times 100$$

Where,  $w_1$  represents the weight of the test fungus in control flasks and  $w_2$  with the bacterial antagonists (Trivedi and Pandey, 2006).

### Elucidation of antagonistic traits

#### Volatile antifungal compounds

The production of volatile antifungal compounds by the isolates was assayed by a sealed plate method by Fiddman and Rossal (1993). On a lawn of test bacteria a second Petri dish containing PDA inoculated with a 6-mm plug of the test fungus was placed over the bacterial culture, each culture was replicated thrice. The two plates were sealed together with parafilm and further incubated at 25°C. Radial growth of the test fungus was measured over 24 h intervals for a period of 5 days.

#### Diffusible antifungal metabolites

Production of diffusible antifungal metabolites was assayed by the method of Montealegre et al. (2003). PDA plates covered with a cellophane membrane were overlaid with nutrient agar and inoculated with 100 µl of bacterial suspension. After incubation for 72 h at 28°C, the membrane along with the grown bacterial growth was removed and an 10 mm disc of a pure culture of *F. oxysporum* was placed in the centre of the plate and incubated at 28°C. The growth of the fungal culture was measured up to a week and compared with growth in the control.

### Microbial siderophore production

#### Qualitative detection of siderophore (plate assay)

Siderophore production by bacterial isolates was detected by the universal method of Schwyn and Neilands (1987) using chrome azurol S (CAS). Cultures positive for siderophore produced an orange halo around the colony where siderophores had chelated iron that had been bound to the dye.

#### Detection of catechol and hydroxamate type siderophores

Catechol-type siderophores was detected and estimated in culture supernatant by Arnow (1937). Hydroxamate-type siderophores was detected and estimated in culture supernatant by Csaky (1948) assay.

#### Production of HCN

All the isolates were screened for the production of hydrogen

cyanide as per the method described by Bakker and Schippers (1987). Petri plates containing 10% trypticase soya agar supplemented with 4.4 g of glycine per litre were inoculated with the bacteria and inverted with a lid containing filter paper, impregnated with 0.5% picric acid and 2% sodium carbonate, over each Petri plate. The plates were incubated at 28°C for 3 to 5 days. A change in color from yellow to orange-brown on the filter paper indicated cyanide production.

### Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water (Cappuccino and Sherman, 1992).

### Evaluation of bioantagonistic potential under glass house condition

A bagculture experiment was conducted to study the influence of the 5 selected antagonists (on the basis of relative antagonistic traits) as seed treatment on seedling emergence, wilt control and growth of chickpea. The polyethylene bags (15 x 10 cm) were filled with sterilized soil inoculated with 1 g fresh weight of fungus at 100 g soil<sup>-1</sup>. Two chickpea (desi) genotypes, JG-62 (susceptible to *Fusarium* wilt) and GPF-2 (wilt tolerant) (Department of Plant Breeding and Genetics PAU, Ludhiana, India) were selected. The seeds of both varieties of chickpea were surface sterilized with 0.1% mercuric chloride and rinsed thoroughly with sterilized water then dipped in the 5 PGPR cultures (10<sup>6</sup> ml<sup>-1</sup> broth) individually and in combination with *Mesohizobium* for half an hour before sowing of seeds. After germination and seedling emergence the bags were watered regularly to maintain optimum moisture and other routine care was taken during the experimentation. Each treatment was replicated 5 times with 10 seeds/ bag, seeds treated with fungicide captan@2 g/kg seeds were sown similarly and parameters for disease incidence in terms of wilting and yellowing of leaves and percentage wilt incident was calculated. The plant growth in terms of percent germination, plant height was recorded up to 60 days after sowing. The statistical analysis was done using CPCS1 software developed by Department of Mathematics, Statistics and Physics, PAU, Ludhiana.

## RESULTS AND DISCUSSION

### Isolation and biochemical characterization of rhizobacterial isolates

A total of 40 rhizobacterial isolates were isolated from chickpea rhizospheric soil from 20 different locations. Out of these 40 isolates, 22 were selected from Kings B medium and 20 of these showed yellowish green pigment whereas two of them showed blue green pigmentation, characteristic of *Pseudomonas* sp., 16 isolates selected from NA medium showed typical colony morphology which was predominantly off-white to creamish in colour, typical of genus *Bacillus* whereas two of the isolates produced pink color pigmentation with entire margin representing genus *Serratia*. The predominance of *Pseudomonas* and *Bacillus* sp. in legume rhizosphere has been reported by many workers. Parmar and Dadarwal (2001) reported that the fluorescent pseudomonads and spore forming *Bacillus* group predominantly colonise the rhizosphere of healthy chickpea plants. Yadav et al. (2010) reported that

population of *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia* and *Serratia* dominated the chickpea rhizosphere. Joseph et al. (2007) in their studies for characterization of PGPR associated with chickpea reported that out of 150 isolates, 40 belonged to genus *Bacillus*, 35 to *Pseudomonas*, 40 to *Azotobacter* and 35 to *Rhizobium*. All the isolates were assessed for cultural, morphological and biochemical characteristics as per Bergey's manual of systematic bacteriology. Sixteen (40%) of the isolates were found to be Gram-positive, rod-shaped bacteria and were found to show profuse growth on NA media at 28°C. Most of the *Bacillus* isolates were positive for indole, Voges-Proskauer, catalase, nitrate reductase and citrate but were negative for methyl red test. Most of them were positive for starch hydrolysis. Twenty two (55%) of the isolates isolated from chickpea rhizosphere showed rapid growth on King's B media at 28°C in 48 h with production of fluorescent yellow to green pigment but two out of these cultures produced dark blue coloured pigment. All were Gram negative rods, indole, methyl red, Voges-Proskauer negative, citrate and catalase positive. They were also able to hydrolyze starch and reduce nitrates. Two of the isolates were Gram negative rod and showed a characteristic production of red coloured pigment on NA media. These pigment producing isolates were positive for Voges-Proskauer, citrate and catalase and nitrate reduction test and Negative for methyl red test (Table 3). On the basis of these tests, the isolates were tentatively placed into three genera: *Bacillus* (1B-16B), *Serratia* (17-S and 18S) and *Pseudomonas* (19P-40P).

### In vitro screening for antagonistic potential

Out of the 40 isolates tested against *F. oxysporum* f. sp. *ciceris* in dual culture under *in vitro* conditions, fifteen isolates of *Pseudomonas* spp., eleven of *Bacillus* spp. and both the *Serratia* isolates showed antagonistic potential against *F. oxysporum*. However variation in inhibition potential was observed although inoculum load was same for all isolates. Out of positive antagonists 2B, 38P, 12B, 7B and 4B isolates showed maximum percent inhibition of test fungus (Figure 1).

As compared to the control, zone of inhibition was clearly visible at 5<sup>th</sup> day after incubation. The percent growth inhibition was found to range between 18.2-41.8% (Table 1). Kumar et al. (2010) reported that *Sinorhizobium fredii* KCC5 and *P. fluorescens* LPK2 inhibited the growth of plant pathogenic fungus *Fusarium udum* in dual culture and increase in fungal inhibition corresponded to incubation period. Strains KCC5 and LPK2 inhibited *F. udum* by 56 and 83% after 6 days of incubation. Similar findings recorded by Kaur et al. (2007), reported that 14 out of 96 *Pseudomonas* isolates from chickpea rhizosphere were highly antagonistic to *F. oxysporum* sp. Growth inhibition of *F. oxysporum* may be due to fungistatic effect or might be attributed to the secretion of antibiotics by the fungi or



**Figure 1.** Growth inhibition of *F. oxysporum* by rhizobacterial isolates.

**Table 1.** Growth inhibition of *F. oxysporum* by rhizobacterial isolates.

Isolate	Growth Inhibition (%)	Isolate	Growth Inhibition (%)
Control	-	20P	18.2
2B	41.8	21P	23.6
4B	32.7	22P	18.2
5B	27.2	25P	23.6
6B	25.4	26P	21.8
7B	32.7	27P	27.3
8B	27.3	28P	30.9
12B	32.7	29P	27.3
13B	25.4	32P	23.6
14B	23.6	34P	32.7
15B	27.3	35P	23.6
16B	21.8	36P	20.0
17S	20.0	37P	23.6
18S	23.6	38P	38.2
-	-	39P	29.1

other inhibitory substances produced by the antagonists.

#### Quantitative evaluation of antagonism

The maximum percent biomass inhibition on dry weight basis was recorded after 5 days of incubation, 2B showed maximum inhibition (93.9%) followed by isolates 34P (84.4%), 28P (79.8%) and 20P (79.8%) (Table 2). Hassanein et al. (2009) reported 75% reduction in dry weight of *F. oxysporum* by *Pseudomonas aeruginosa*. Trivedi and Pandey (2006) also recorded 41% reduction in biomass of *F. oxysporum* by *Pseudomonas corrugata* in liquid media. All the isolates showed different ability to inhibit mycelial growth of the fungus and a notable reduc-

tion in mycelial biomass was observed as compared to the control. *In vitro* broth-based dual cultures offer a better method for evaluation of antagonistic efficiency of the biocontrol agents as the liquid medium provides a better environment to allow the antagonistic activities from all possible interacting sites.

#### Elucidation of antagonistic traits

All the 28 antagonistic rhizobacterial isolates were found to produce toxic volatiles. A reduction in the radial growth of the test fungus *F. oxysporum* sp. *ciceris* was observed after 120 h of incubation due to production of volatile anti-fungal compounds (Figure 2). However growth inhibition



**Table 2.** Inhibition in mycelial proliferation of *F. oxysporum* by rhizobacterial isolates.

Isolate	Reduction in dry weight (%)	Isolate	Reduction in dry weight (%)
Control	-	20P	74.8
2B	93.9	21P	64.8
4B	66.0	22P	69.9
5B	61.1	25P	15.4
6B	40.2	26P	66.0
7B	73.7	27P	69.2
8B	33.4	28P	79.8
12B	70.6	29P	72.4
13B	15.2	32P	29.6
14B	74.1	34P	84.4
15B	14.9	35P	83.7
16B	24.0	36P	30.6
17S	61.3	37P	26.6
18S	67.4	38P	74.1
-	-	39P	67.7

varied between 14.2-64.2% which was in accordance with findings of the dual culture studies where some isolates showed varying antagonistic potential. Maximum inhibition was observed in 15B isolate (64.2%) after 5 days of incubation (Table 3). Arafoui et al. (2006) reported that 8 of the 21 rhizobacterial isolates from chickpea rhizosphere significantly inhibited the growth of *F. oxysporum* sp. *ciceris* by producing volatiles. The production of volatile antifungal compounds by *Pseudomonas cepacia* and fluorescent pseudomonads has also been reported by other workers (Tripathi and Johri, 2002; Fernando et al., 2006).

### Production of diffusible antifungal compounds

Diffusible antimetabolites are the non-volatile antibiotics produced by bacteria in low concentrations such as phenazine-1-carboxylic acid, 2-hydroxyphenazines, phenazine-1-carboxamide (PCN), etc. and such compounds are implicated in disease suppression in plants (Kim et al., 2011). All the isolates produced toxic diffusibles. A notable reduction in the radial growth of *F. oxysporum* sp. *ciceris* was observed after 120 h of incubation due to diffusible antifungal metabolites (Figure 3). Growth inhibition varied between 22.2 - 77.8%, maximum being observed with 39P (77.8%) after 5 days of incubation (Table 4). Thus, these isolates produced both volatiles as well as diffusible antifungal metabolites which are strongly implicated in antifungal activity. Tjeerdvan et al. (2004) in their studies reported that *P. fluorescens* produces a broad-spectrum antibiotic phenazine-carboxylic acid (PCA), which is active against a variety of fungal root pathogens.

### Production of siderophore

Nineteen of the antagonistic rhizobacteria showed a

distinct orange halo on CAS plates indicating siderophore production. Out of these eleven belonged to *Pseudomonas* spp., seven to *Bacillus* spp. and one belonged to *Serratia* spp. Siderophore production was found to start after 24 h of incubation, reaching a maximum after 72 h, when organism had entered into stationary phase. Maximum siderophore production was shown by 38P followed by 34P and 28P. Gupta and Goel (2002) reported similar results on CAS agar where diameter of halo ranged from 0.72-2.6 cm after 24-72 h of incubation. In a similar study conducted by Akhtar and Siddiqui (2009), siderophore production by *Pseudomonas* sp. isolated from chickpea rhizosphere was reported and the halos formed on CAS medium ranged from 1.6-1.7 cm. The results of these workers are in corroboration with the present study.

Out of the 19 isolates producing orange halo on CAS plates, 14 were found to produce catechol-type siderophore which ranged from 21.2-93.2  $\mu\text{g.ml}^{-1}$  (Table 5). Highest amount of catechol type siderophore was produced by 34P (93.2  $\mu\text{g.ml}^{-1}$ ) followed by 38P (92.4  $\mu\text{g.ml}^{-1}$ ), 7B (89.2  $\mu\text{g.ml}^{-1}$ ) and 28P (82.1  $\mu\text{g.ml}^{-1}$ ). However, isolate 34P and 2B also produced higher amount of hydroxamate type siderophore (65.2  $\mu\text{g.ml}^{-1}$  and 63.5  $\mu\text{g.ml}^{-1}$ ), respectively whereas overall hydroxamate type siderophores were produced by only 12 isolates. Maximum production was recorded in isolate 26P (100.2  $\mu\text{g.ml}^{-1}$ ) followed by 38P (70.7  $\mu\text{g.ml}^{-1}$ ), 34P (65.2  $\mu\text{g.ml}^{-1}$ ) and 29P (63.5  $\mu\text{g.ml}^{-1}$ ) (Table 5). Only seven of the isolates produced both types of siderophores. In a study conducted by Siddiqui et al. (2007), it was found that *Pseudomonas putida* caused greater root colonization and greater siderophore production than the other species of PGPR. Sayyed et al. (2005) found that *P. putida* and *P. fluorescens* produced 83 and 87% units of siderophores in succinate media.



**Figure 2.** Antagonistic effect of volatile antifungal compounds on growth of *F. oxysporum*.

**Table 3.** Effect of volatile antifungal metabolites on growth of *F. oxysporum*.

Treatment	Growth inhibition (%)	Treatment	Growth inhibition (%)
Control	-	20P	42.8
2B	30.0	21P	20.0
4B	28.6	22P	31.4
5B	35.7	25P	28.5
6B	40.0	26P	38.5
7B	44.3	27P	27.1
8B	14.2	28P	42.8
12B	21.4	29P	35.7
13B	28.5	32P	34.2
14B	40.0	34P	50.0
15B	64.2	35P	28.5
16B	38.6	36P	30.0
17S	21.4	37P	25.7
18S	30.0	38P	35.7
-	-	39P	30.0



**Figure 3.** Antagonistic effect of diffusible antifungal compounds on growth of *F. oxysporum*.

**Table 4.** Effect of diffusible antifungal metabolites on growth of *Fusarium oxysporum*.

Isolate	Growth inhibition (%)	Isolate	Growth inhibition (%)
Control	-	20P	33.3
2B	22.2	21P	48.8
4B	37.8	22P	44.4
5B	37.8	25P	22.2
6B	31.1	26P	33.3
7B	24.4	27P	15.5
8B	37.8	28P	66.6
12B	31.1	29P	71.1
13B	28.9	32P	44.4
14B	37.8	34P	44.4
15B	42.2	35P	31.1
16B	33.3	36P	42.2
17S	42.2	37P	48.9
18S	48.9	38P	53.3
-	-	39P	77.8

**Table 5.** Production of catechol and hydroxamate-type siderophore by rhizobacterial isolates.

Isolate	Catechol type siderophore* ( $\mu\text{g.ml}^{-1}$ )	Hydroxamate type siderophore* ( $\mu\text{g.ml}^{-1}$ )
2B	73.6	28.1
4B	65.4	-
5B	21.2	-
7B	89.2	33.4
8B	32.7	40.6
12B	-	48.8
14B	-	51.9
18S	25.4	43.2
21P	49.4	-
22P	68.2	-
26P	-	100.2
28P	82.1	-
29P	-	63.5
34P	93.2	65.2
36P	59.5	-
37P	53.2	-
38P	92.4	70.7
39P	56.2	50.4
40P	-	46.5

### Production of HCN

Out of 28 antagonistic bacterial isolates only twelve of the isolates were found positive for HCN production, of which seven belonged to *Pseudomonas* spp. and five belonged to *Bacillus* spp. Isolates 3B, 9B, 12B, 20P, 27P, 29P, 34P, 38P and 39P were found to be strong producer of HCN, causing colour change from yellow to reddish brown whereas 4B, 6B and 25P were moderate producers indicated by orange brown colour. Siddiqui and Shakeel (2009) reported HCN production by twenty-one *Pseudomonas*

isolates from pigeonpea rhizosphere, out of which three were potent producers of HCN, whereas eleven were moderate producers. Saraf et al. (2008) isolated 10 strains of *Pseudomonas* from chickpea rhizosphere, out of which three produced HCN, the strongest producer being *Pseudomonas* M1P3.

### Production of $\text{NH}_3$

All the antagonistic isolates were found to be ammonia

**Table 6.** Effect of bioantagonist on germination, plant growth and disease control under glass house conditions in chickpea variety (JG-62 and GPF-2).

Treatment	Seedling emergence (%)		Shoot length (cm)		Incidence of wilt (%)	
	JG-62	GPF-2	JG-62	GPF-2	JG-62	GPF-2
Negative control	66.6	69.0	85.0	14.5	85.0	74.4
Captan (fungicide)	75.0	68.3	55.5	16.4	55.5	33.7
2B	88.3	88.3	60.3	18.0	60.3	39.6
7B	88.3	83.3	56.6	18.4	56.6	40.0
28P	83.3	81.7	68.0	18.3	68.0	42.8
34P	81.6	90.0	47.7	19.0	47.7	40.7
38P	93.3	91.7	44.6	19.1	44.6	38.1
2B+ <i>Rhizobium</i>	86.6	93.3	36.5	21.5	36.5	26.7
7B+ <i>Rhizobium</i>	78.3	85.0	59.5	18.6	59.5	33.3
28P+ <i>Rhizobium</i>	85.0	86.7	56.8	20.7	56.8	38.4
34P+ <i>Rhizobium</i>	83.3	95.0	40.0	20.5	40.0	29.8
38P+ <i>Rhizobium</i>	81.6	93.3	36.7	19.6	36.7	30.3
CD at 5%	1.32	1.10	1.68	1.26	1.68	1.56

producers. Out of these, 11 were *Bacillus* isolates, 15 were *Pseudomonas* isolates and both the *Serratia* isolates. A marked variation in the ability to produce ammonia was observed amongst the isolates as indicated by the intensity of the color developed. Isolates 17S, 18S, 21P, 32P, 34P, 37P, 38P and 39P were strong producers of ammonia. Ammonia production was detected in 95% of the isolates from the rhizosphere of rice influencing plant growth promotion (Samuel and Muthukkaruppan, 2011). Joseph et al. (2007) reported ammonia production by 95% of *Bacillus* isolates and 94.2% of *Pseudomonas*. Mishra et al. (2010) also reported that *B. subtilis* MA-2 and *P. fluorescens* MA-4 were found to be efficient in ammonia production and significantly increased biomass of medicinal plant Geranium.

#### Evaluation of wilt control and plant growth promoting potential under glass house condition

##### Effect of rhizobacteria on seedling germination

The effects of seed treatment with PGPR and PGPR + *Mesorhizobia* on two varieties of chickpea, JG-62 and GPF-2 revealed a synergistic interaction between the inoculants as evidenced by seedling emergence. An enhancement in percent germination was observed in treatments comprising of PGPR + *Mesorhizobia* as compared to PGPR alone that also has shown some degree of enhancement in percent germination. Most of the rhizobacterial isolates enhanced seedling emergence, as compared to negative control as well as fungicide (captan) treatment. Maximum seedling emergence was recorded in 38P (93.3 and 91.7% in JG-62 and GPF-2 varieties, respectively). However, combination of bioantagonist with *rhizobia* further enhanced the seedling emergence in the present study (Table 6). Similar findings have been repor-

ted by Ashrafuzmann et al. (2009). Their studies also revealed that treatment with PGPR strains improved germination rate of the seeds as compared to negative control in the case of rice and maize, respectively. However, Hahm et al. (2012) reported 100% seed germination of pepper on treatment with PGPR culture alone. The increase in seed germination percentage may be due to modulation of hormone-linked phenomenon such as auxins and gibberellins production. Relative seedling emergence between different treatments and between both the varieties (GPF-2 and JG-62) has been shown in Figure 4 and Figure 7 respectively..

##### Effect of rhizobacteria on incidence of wilt

The pot studies, with 5 bioantagonists 2B, 7B, 28P, 34P and 38P exhibited similar results as *in vitro* tests, providing further confirmation of the efficacy of these isolates in suppressing wilt in chickpea. Symptoms of wilting observed were dry, brown and drooped plantlets whereas non-wilted plants were fresh and green. Previous studies also have reported antagonistic activity of *Pseudomonas* sp. against *Fusarium* sp. (Leon et al., 2009). The degree of disease suppression was significantly increased where dual culture treatment was used. However, overall more wilting was observed in susceptible variety JG-62 than resistant variety GPF-2. Hahm et al. (2012) also reported that the disease protection conferred by mixture of strains was not significantly different than that with the single strains. In both varieties (GPF-2 and JG-62), maximum reduction in wilt incidence was shown by 38P followed by 34P and their combination with *Rhizobium* as compared to fungicide (Table 6, Figure 5).

Variety JG-62 exhibited 85% wilt incidence in the negative control. However, seed treatment with fungicide



**Figure 4.** Relative seed emergence between different treatments.



**Figure 5.** Relative symptoms of wilting between different treatments.

reduced the disease severity to 55.5% (Table 6). Treatment with bioantagonist 38P showed least disease incidence (44.6%) followed by 34P (47.7%), 7B (56.6%), 2B (60.3%) and 28P (68%). Isolates 2B (60.3%) and 28P (68%) showed disease severity as compared to other 3 isolates 38P, 34P and 7 B. Comparatively lesser disease severity was observed in the variety GPF-2 (Table 6).

However in variety GPF-2, least disease incidence was scored by 38P (38.1%) followed by 2B (39.6%) and 7B (40%). In both varieties (GPF-2 and JG-62), further reduction in wilt incidence was recorded in the case of co-inoculation with PGPR and *Rhizobium* due to their synergistic nitrogen fixation and antagonistic effect. Rhizobacterial isolates 38P, 34P and 7B exhibited maximum reduction of *Fusarium* wilt and this could be attributed to their ability to produce volatile and diffusible antifungal metabolites, phenolic compounds, cyanides and siderophores.

#### Effect of rhizobacteria on plant growth

Chickpea variety JG-62 treated with only PGPRs showed taller plants in the case of 4 isolates, that is, 38P (19.2

cm), 34P (18.8 cm), 2B (18.2 cm) and 28P (18.1 cm) which was at par with the plant height recorded with fungicide treatment but significantly higher than negative control (15 cm) (Table 6 and Figure 6). Co-inoculation of PGPR and *Rhizobium* resulted in further significant increase in shoot length as *Rhizobium* are reported to produce plant growth regulators such as auxins, cytokinins and gibberellins like substances that stimulate and enhance plant growth (Hemissi et al., 2011). Synergistic effect in the use of dual cultures is well documented (Hahm et al., 2012). Comparatively, in GPF-2 variety also, all the five PGPR, that is, 38P (19.1 cm), 34P (19 cm), 7B (18.4 cm), 28P (18.3cm) and 2B (18 cm) enhanced shoot length significantly, maximum being with 38P (19.1 cm) as compared to fungicide treatment (16.4 cm) and negative control (14.5 cm) (Table 6). In both varieties, that is, GPF-2 and JG-62, further increase in shoot length was recorded in the case of co-inoculation with PGPR and *rhizobium* as compared to PGPR isolates alone (Table 6) and in both varieties, rhizobial isolates 38P, 34P and 28P exhibited maximum enhancement of shoot length and this could be attributed to their ability to produce siderophore that help the plants not only to sequester iron from soil but also inhibit the fungal pathogen and thus enhance

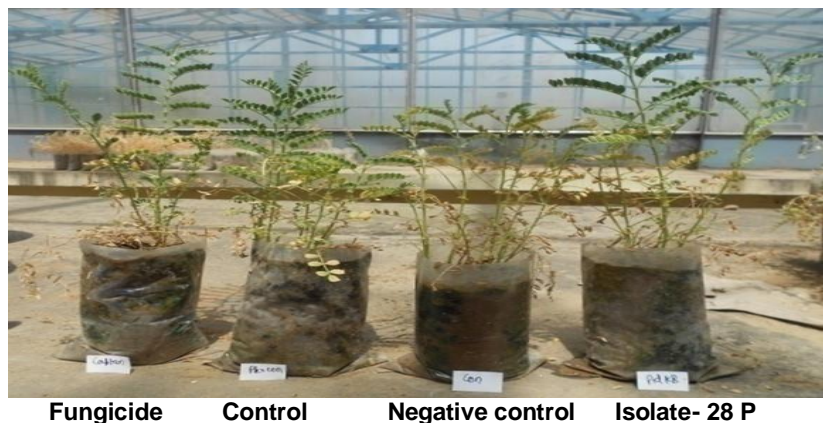


Figure 6. Relative growth between different treatments (GPF-2).

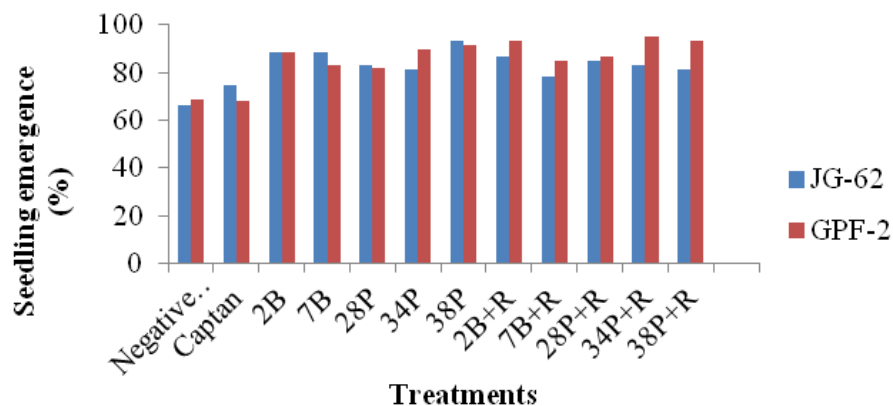


Figure 7. Relative seed emergence between different treatments in JG-62 and GPF-2 variety.

the growth of plants significantly. A relative comparison of plant growth is depicted in Plate 10. Hahm et al. (2012) also reported in his studies that PGPR treatments resulted in the taller peppers as compared to negative control treatments.

It has been reported that even if mixtures of PGPR strains do not always results in additive or synergistic effects of possible advantage, different strains may have different mechanisms and their combinations may provide a spectrum of activity which may have a beneficial effect on plant health and productivity (Raupach and Kloepper, 2000).

This study provided an initial assessment of the potential of rhizosphere bacteria associated with chickpea in Punjab, Uttar Pradesh and Palampur to control wilt of chickpea and promote plant growth individually and in combination with *Rhizobium* under controlled conditions in glass house. Three isolates, that is, 38P, 34P and 28P appeared to be the most promising biological control agents against chickpea wilt. But further study of these antagonists under field condition is needed for the more evident confirmation of their antagonistic trait against

fusarium wilt in chickpea.

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Full Length Research Paper

## Accumulation of some heavy metals by metal resistant avirulent *Bacillus anthracis* PS2010 isolated from Egypt

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The bacteria with a high growth rate were isolated from polluted industrial waste water. The bacteria *Bacillus anthracis* PS2010 have variable resistant to heavy metals such as Cd, Cu, Co, Zn and Pb. Out of which the minimal inhibitory concentrations were 0.6, 2.0, 0.8, 4.0 and 3.0 mM, respectively. The potent bacterium has optimal biosorption capacity raised according to the metal, incubation temperature, pH of the solution and contact time. Under optimal conditions, the bacterium was capable of taking up the heavy metals Cd, Cu, Co, Zn and Pb at 3.41, 2.03, 4.75, 5.22 and 6.44 mg/g dry weight. Transmission electron microscopy showed accumulation of Pb metal external to bacterial cells. The mechanism of heavy metal tolerance in *Bacillus anthracis* PS2010 is chromosomally encoded. *Bacillus anthracis* harbored no plasmid.

**Key words:** Heavy metal uptake, bacterial biosorption, plasmid, *Bacillus anthracis* PS2010.

### INTRODUCTION

Mobilization of heavy metals in the environment due to industrial activities is of serious concern due to the toxicity of these metals in humans and other forms of life. Removal of toxic heavy metals from industrial waste waters is essential from the standpoint of environmental pollution control (Puranik and Pakniker, 1999; Guangyu and Thiruvengkatachari, 2003). Heavy metals mercury (Hg), nickel (Ni), lead (Pb), arsenic (As), zinc (Zn), cadmium (Cd), aluminum (Al), platinum (Pt), copper (Cu) and cobalt (Co) are trace metals with a density of at least five times that of water, they are stable elements (meaning they cannot be metabolized by the body) and bio-accumulative (passed up the food chain to humans). These include: Hg, Ni, Pb, As, Zn, Cd, Al, Pt, Cu and Co. Some heavy metals have function in the body while others can

be highly toxic for human health (Parry, 2009; Hornung et al., 2009). Toxicity of metallic ions could be the result of competition with or replacing a functional metal as well as causing conformational modification, denaturation, and inactivation of enzymes and disruption of cellular and organelles integrity (Blackwell et al., 1995).

Remediation technologies using microorganisms are feasible alternatives to the physical cleaning of soil or the concentration of metals in polluted water by physical or chemical means (Valls and de Lorenzo, 2002; Abou Zeid et al., 2009; Adewole et al., 2010). Metal tolerance reflects the ability of an organism to survive in an environment with high concentration of metals or to accumulate high concentration of metal without dying. Metal exposure also leads to the establishment of tolerant microbial populations,

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which are often represented by several Gram positives belonging to *Bacillus*, *Arthrobacter* and *Corynebacterium*, as well as Gram negatives as *Pseudomonas*, *Alcaligenes*, *Ralstonia* and *Burkholderia* (Kozdro and Van Elsas, 2001; Ellis et al., 2003; Ajaz et al., 2010). In contaminated sites, these populations may be involved in the alteration of mobility of metals through their reduction, accumulation, and *in situ* immobilization by extracellular precipitation (Roane, 1999). Different microorganisms such as fungi, yeast and bacteria were tested for the availability and biosorption potential to bind heavy metals (Volesky and Holan, 1995). There are at least three types of microbial processes that can influence toxicity and transport of metals and radionuclides: biotransformation, bioaccumulation and biodegradation. However, microorganisms can interact with these contaminants and transform them from one chemical form to another by changing their oxidation state through the addition (reduction) or removal (oxidation) of electrons. Several authors have reported the high capability of heavy metals bioaccumulation by Gram negative bacteria (Noghabi et al., 2007; Choi et al., 2009; El-Shanshoury et al. 2012). Bacterial resistance to heavy metals might be chromosomal or plasmid mediated (Raval et al., 2000; Zouboulis et al., 2003). Zolgharnein et al. (2007) reported that the frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria.

The main objective of this study was to obtain a local bacterium resistant to heavy metals, in order to be used for remediation of metal ions in polluted habitats in Egypt. In this connection, the potential of *Bacillus anthracis* PS2010 to absorb and uptake Cd, Cu, Co, Zn, and Pb was focused on, with special emphasis on Pb. The mechanism and the form of Pb accumulation by the bacterium are discussed.

## MATERIALS AND METHODS

### Source of bacterial isolate

The tested isolate, *B. anthracis* PS2010 (accession no. HQ856038) used in this study was isolated previously from location polluted with heavy metals in Egypt. The isolate was identified by sequencing 16S rRNA gene and comparing the sequences with database library using analysis software. The program Blast was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny was performed using BioEdit software (Hall, 1999).

### Samples collection

Samples were collected from 3 different polluted locations: a lathe and motor manufacturing workshops in Tanta, El-Gharbia Governorate, and industrial wastewater of Industrial Area in Quesna, El-Monofeya Governorate. The samples included dusts containing metal filings, grinding metals and industrial wastewater.

### Isolation of bacteria

The isolation of bacteria was carried out on nutrient agar medium

adjusted at pH  $7.3 \pm 0.2$ . One gram of each dry contaminated dust was dissolved in 50 ml sterile distilled water, and then 250  $\mu$ l from the resultant suspension were spread on the surface of nutrient plates. The plates were incubated for 24 h at 35°C. Grown colonies were investigated for their morphological characteristics, purified and kept at 5°C as slant cultures.

### Isolation and selection of metals resistance bacteria

All the isolated bacteria were checked for their metal tolerance against five selected metals: Cd, Cu, Co, Zn and Pb. Aqueous solutions of the metal salts: CdCl<sub>2</sub>·2H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, CoCl<sub>2</sub>·7H<sub>2</sub>O and Pb(NO<sub>3</sub>)<sub>2</sub> were prepared in de-ionized water and 0.1 mM/L were added to the nutrient agar medium. Media were sterilized, dispensed in Petri plates and then inoculated with bacteria; the plates were incubated at 35°C for 24 h. The tolerance was checked on the basis of growth observed within 24 h of cultivation according to Chowdhury et al. (2008). The grown colonies were used for detection of minimal inhibitory concentrations (MICs) for each metal. The most highly tolerant bacterium (with highest MICs) was selected for identification and further study.

### Preparation of bacterial culture

One liter of nutrient broth medium free of metals was prepared and sterilized by autoclaving. Medium was inoculated with 24-h-old *B. anthracis* PS2010 culture previously prepared and grown to the end of exponential phase (growth curve data not shown).

### Determination of the minimum inhibition concentration (MIC)

The minimum inhibition concentration was checked for its metal tolerance against five selected metal salts: CdCl<sub>2</sub>·2H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, CoCl<sub>2</sub>·7H<sub>2</sub>O, and Pb(NO<sub>3</sub>)<sub>2</sub>. Aqueous solutions of these salts were prepared in de-ionized water, the pH values of the solutions were adjusted to neutral (pH 7). The flasks containing media and variable concentration of metals were incubated at 35°C with *B. anthracis* PS2010 for 24 h. The highly tolerant isolate (with the highest MICs values) was selected for characterization, identification and further experiments.

### Electron microscopy

The highly uptake isolate (of the five metal ions mg/g dry weight), (with following order: Pb<sup>2+</sup> > Zn<sup>2+</sup> > Co<sup>2+</sup> > Cd<sup>2+</sup> > Cu<sup>2+</sup>) were selected for characterization, identification. Before and after the treatment with Pb(NO<sub>3</sub>)<sub>2</sub>, cell of isolate was examined by scanning electron microscope (SEM) to detect any change in the morphology of the cells as a result of metal treatment. The control and treated cultures were also examined by transmission electron microscopy (TEM) in order to identify the location of lead particles within the cells (Chowdhury et al., 2008). Cells of control and treated cultures (as described before) were centrifuged at 5000 rpm, washed twice and fixed in 2.5% buffered glutaraldehyde in 0.1 M PBS (phosphate buffer solution) pH: 7.4 for 24 h at 4°C, washed three times with PBS for 10 min each time and then centrifuged at 5000 rpm. These steps were followed by post fixation in 1% osmic acid for 30 min. It was dehydrated in a series of ethyl-alcohol (30 to 100%) infiltrated with acetone each concentration for 30 min.

### Transmission electron microscopy (TEM)

After dehydration, samples were embedded in Araldite resin. The plastic molds were cut at 850 nm thicknesses in a LEICA Ultra cut ultra-microtome, and stained with 1% toluidine blue. After examination of semi-thin sections, ultrathin sections were cut at thickness of

75 nm, stained with uranyl acetate for 45 min, then counter stained with lead citrate and examined. Images were taken using a JEOL, JEM-100 SX electron microscope. All the isolated bacteria that were able to grow on media supplemented with different salt decreased gradually by increasing the concentration of each metal salt. The isolate was low tolerant to all metals which showed higher tolerance, in comparison with other isolates. The highly tolerant organism for most metals was selected for characterization, identification and further experiments.

### Penicillin sensitivity

The penicillin sensitivity of the isolate was observed according to Mueller-Hinton (1941). Mueller Hinton agar medium was prepared and sterilized in autoclave at 121°C for 20 min. The medium was suspended in sterilized Petri plates prior to inoculation of the plates with the tested organism and then a sterilized penicillin disc (Bioanalysis, 10 U) was placed in the centre of the plate. The plate was incubated overnight at 35°C. The presence of inhibition zone around the disc was checked.

### Sequencing of 16S-rRNA gene and phylogenetic analysis

The bacterium selected as the most resistant isolate to all five heavy metals was identified and confirmed using Biolog automated system Bochner (1989). The selected isolate was identified by sequencing of 16S-rRNA gene. Bacterial genomic DNA was extracted from the cells by using Qiagen kit. The DNA was used as template for PCR using universal primers. The forward primer is 5'-AAC TGG AGG AAG GTG GGG AT-3'. The purified product of the PCR is used as template in cycle sequencing using 3130 X / Genetic Analyzer, Applied Biosystems, Hitachi, Japan, with Big dye terminator cycle sequences technique, developed by Sanger et al. (1977). The products were purified using special column. The sample became ready for sequencing in 3130 X DNA sequencer and analysis. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999).

### Plasmid isolation

To prove the tested organism is avirulent, the plasmid DNA of the selected isolate was extracted, purified and separated using agarose gel electrophoresis according to the method employed by Manniatis et al. (1982), for isolation and screening plasmid. The developing bands were compared with DNA marker.

### Pathogenicity of the isolated *Bacillus anthracis* PS2010

In order to detect if our isolated *B. anthracis* PS2010 was pathogenic strain or not, the presence of plasmid(s) coding for the pathogenicity was tested. Plasmid isolation was carried out in The City for Scientific Research and Biotechnology Applications, New Borg EL-Arab City, Alexandria, Egypt. The plasmid was tested by using Qiagen kit, the QIAprep miniprep procedures use the modified alkaline lysis method of Birnboim and Doly (1979), followed by adsorption of DNA onto silica in the presence of high salt.

### Optimization of metal uptake

#### Effect of different incubation temperature

One milliliter of the aliquots of *B. anthracis* PS2010 selected isolate suspension, 10 h old (exponential phase) were inoculated in 100 ml nutrient broth media containing sub-MICs concentrations of CdCl<sub>2</sub>·2H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, CoCl<sub>2</sub>, ZnSO<sub>4</sub>·7H<sub>2</sub>O, and Pb(NO<sub>3</sub>)<sub>2</sub> respectively.

After the addition of metal solutions, the media was adjusted at pH=7 by using 0.1 N NaOH and 0.1 N HCl and (0.1 N HNO<sub>3</sub> with Pb (NO<sub>3</sub>)<sub>2</sub>). The cultures were incubated at different temperatures (25, 35 and 45°C) for 24 h. The incubated cultures were centrifuged at 5000 rpm for 20 min. The supernatants were used for the determination of the residual metal ion contents by using atomic absorption spectrophotometer (Perkin Elmer 2380) with hollow cathode lamp at specific wavelength for each metal. Control cultures without the inoculation of bacteria were prepared to detect the initial metal concentration.

#### Effect of different pH values

To test the pH effect of nutrient broth media containing metal solutions, the solution pH were adjusted at different values (2, 5, 7, 8 and 9). All cultures were incubated at 35°C for 24 h. The initial and the residual metal concentrations were measured.

#### Effect of contact times

Media containing metal solutions adjusted at pH=7 and inoculated with selected isolate was incubated at 35°C for different periods (12, 18, 24 and 48 h). The initial and residual concentrations were measured as mentioned earlier.

### Determination of metal uptake by the resistant bacteria

The uptake of Cd, Cu, Co, Zn and Pb metals in mg/g dry wt. were detected. According to each metal, bacterial culture (10 h old) was adjusted at the optimal pH, incubated temperatures and optimal period of time. The cultures were centrifuged at 5000 rpm for 20 min. The supernatants were discarded and the residual bacterial pellets were washed with sterilized distilled water and then the bacterial biomasses were transferred to known weight. The supernatants were used for the determination of the residual metal ions contents in mg/L. The initial metal ions contents in mg·l<sup>-1</sup> were determined in control without bacterial cell. Supernatants were passed through bacterial filters (0.22 µm diameter). The determinations were undertaken by using Atomic Absorption spectrophotometer (model Perkin Elmer 2380) (Abou Zeid et al., 2009). The metal uptake in mg/g dry wt. was calculated according to the equation of Volesky and May-Phillips (1995):

$$\text{Metal uptake (mg/g)} = V (C_i - C_f) / w$$

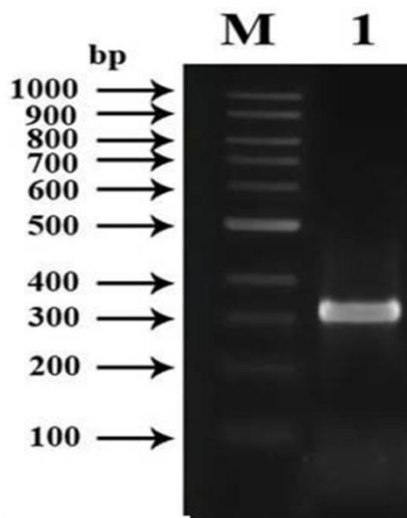
Where, C<sub>i</sub> = initial metal concentration (mg/L), C<sub>f</sub> = final metal concentration (mg/L), V = volume of reaction (L), W = total biomass (g).

### Statistical analysis

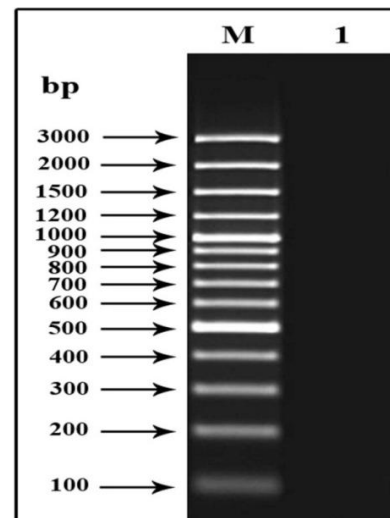
The statistical analysis was carried out using SAS program version 6.12. Data obtained were analyzed statistically to determine the degree of significance between treatments using one way analysis of variance (ANOVA) by the methods described by Cochran and Cox (1960).

## RESULTS AND DISCUSSION

The pure isolated strain obtained from the polluted location was studied. Different concentrations of each metal solution were prepared, the minimum concentration of each metal added was 0.1 mM/L and the concentration was gradually increased till MIC was achieved. The isolated strain was found to give low tolerance with CdCl<sub>2</sub> and



**Figure 1a.** Agarose gel electrophoresis for PCR products of 16S-rRNA gene analysis M: 1 kbp DNA marker, 1: PCR product of *B. anthracis* PS2010.



**Figure 1b.** Agarose gel electrophoresis of plasmid profile for *B. anthracis* PS2010. M: DNA marker, 1: Plasmid of *B. anthracis* PS2010.

was found to be highly tolerant to  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . The MICs of  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$  were 0.6, 2.0, 0.8, 4.0 and 3.0 mM/L, respectively. This varying response of tested bacteria might be due to variation in resistance mechanisms (Abou Zeid et al., 2009).

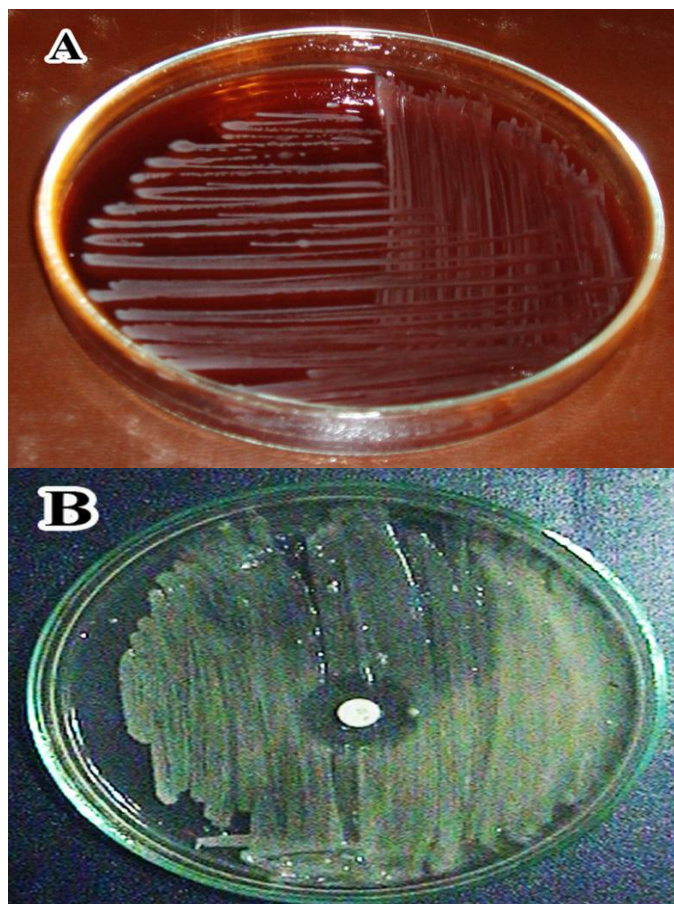
For phylogenetic analysis, the 16S rRNA gene sequence of a single band of MW (~320 bp) was obtained (Figure 1a) when compared with those retrieved from Gen Bank database. The sequences have high similarity or are even identical to cultivable bacterial organism. The phylogenetic analysis of the 16S rRNA gene partial sequence of isolated strain revealed close similarity with *B. anthracis* TC-3, *B. anthracis* A0248, *Bacillus thuringiensis* BMB171 and *Bacillus cereus* LS24 (96% similarity) (Figure 2). Wang and Chen (2006), reported that the members of *B. cereus* group share many of their biochemical, morphological and they are very closely related in gene sequence based on their 16S rRNA. According to Health Protection Agency in UK (2007), for the identification of *Bacillus* species, the differentiation between *B. cereus* members depends on 3 main tests: penicillin sensitivity, motility and hemolytic activity. Since our isolate was penicillin sensitive, non-motile, with non-hemolytic activity and characteristic grayish white colonies on blood agar (Figure 3), it was identified as *B. anthracis*.

Resistance to heavy metals might be chromosomal or plasmid mediated (Gupta et al., 1999). Zolgharnein et al. (2007) reported that the frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria. So, it is important to get safe bacteria for possible application in metal bioremediation. Virulent strains of *B. anthracis* harbor two endogenous plasmids, pXO1 and pXO2 which code for the major known virulence factors of this organism (Thorne, 1985).

Figure 1b revealed the absence of plasmid DNA in extracts, indicating that this strain was avirulent. A virulent *B. anthracis* strain which lack these plasmids (pXO1, pXO2) have also been found and they appear to be very similar to *B. cereus* and other related species unless tests for bacteriophage susceptibility, motility and hemolysis are performed (Henderson et al., 1994). The *B. anthracis* strain used in this study found to lack both plasmids (pXO1, pXO2), thus it was regarded as avirulent strain and safe for bioremediation purposes. It was thus submitted to Genbank as *B. anthracis* PS2010 with accession no. HQ856038. In agreement, Silver (1996), reported that bacterial cells encoded resistance systems for several toxic metal ions including  $\text{Ag}^+$ ,  $\text{As}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Cr}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Sb}^{3+}$ ,  $\text{Te}^{2+}$  and  $\text{Zn}^{2+}$ . Resistance to heavy metals might be mediated by genes encoded on chromosomes, plasmids or transposons (Tenover and McGowan, 1996; Ghosh et al., 2000). These chromosomes carried genes responsible for resistance to high levels of toxic heavy metals ( $\text{As}^{2+}$ ,  $\text{Cr}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$ ) as well as ampicillin antibiotic. The ability to grow in the presence of  $\text{Pb}^{2+}$  was seen in chromosome encoded (Wasi et al., 2008).

The capacity of living cells to remove metal ions from aqueous solutions is also influenced by environmental growth conditions, as temperature, pH and biomass concentrations (Chen and Ting, 1995). In the present study, the growth and metal uptake capability of the resistant *B. anthracis* PS2010 were affected by the different environmental conditions (incubation temperature, pH value and contact time). The effect of different incubation temperatures on the uptake of the five selected metals (Figure 4) revealed that the maximum uptake for  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$  was obtained at 35 and 25°C, respectively. The uptake of  $\text{Zn}^{2+}$





**Figure 3.** Blood agar culture showing grayish-white growth with non-hemolytic activity (A) and penicillin sensitivity test on Mueller-Hinton agar (B) of *B. anthracis* PS2010.

tion temperature to 45°C. Higher temperatures usually enhance sorption due to the increased surface activity and kinetic energy of the solute which could promote the active uptake or attachment of metal to cell surface, respectively (Sağ and Kutsal, 2000; Vijayaraghavan and Yun, 2007). The accumulation of heavy metals by *B. anthracis* PS2010 was found to be decreased by increasing the temperatures to 45°C, these results agree with the results obtained by Mameri et al. (1999), Prescott et al. (2002) and Uslu and Tanyol (2006).

The pH value is one of the main factors in the biosorption efficiency and binding to microorganisms (Babich and Stotzky, 1985; Lopez et al., 2000; Jalali et al., 2002; Pardo et al., 2003). Results indicate that pH 8 was optimum for  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$  and pH range 7-8 was the optimum for  $\text{Cu}^{2+}$  uptake. These results agree with that obtained by Ozdemir et al. (2003). On the other hand, pH 5 was optimum for  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$  uptake by *B. anthracis* PS2010. In the case of  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$ , increasing the alkalinity of the solutions was followed by large decrease in the uptake of these metals especially  $\text{Co}^{2+}$ . By increasing the pH over 5 in the case of  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$ , the uptake started to de-

crease gradually (Ozdemir et al., 2003). In the case of  $\text{Cu}^{2+}$ , the uptake seemed to be slightly increased by increasing the pH from 5 to 7 (Figure 5). Silva et al. (2009) revealed the chromium level sorbed by *P. aeruginosa* AT18 biomass with 100% removal was in pH range 7-7.2. At low pH, the  $\text{Cd}^{2+}$  accumulation was decreased and caused increased competition between hydrogen and cadmium ions for binding sites on the cell surface or by an increase in metal efflux pump activity due to an increase in the proton gradient that drives the efflux pump. The pH 7 was optimum for  $\text{Pb}^{2+}$  and  $\text{Co}^{2+}$  accumulation by *B. anthracis* PS2010, while pH 5 was optimum for  $\text{Zn}^{2+}$  uptake. It was reported that pH 4.5 was optimum for biosorption of lead by *Citrobacter* strain MCM B-181 and pH < 3 or > 5 resulted in lower biosorption efficiency of lead (Puranik and Pakniker, 1999; Guangyu and Thiruvengatchari, 2003). At highly acidic pH (<3) lead ions compete with hydrogen ions on the binding sites of microbial cells. However, at higher pH (>5) solubility of lead was lowered (Chang et al., 1997). The variation in external pH can also affect the degree of protonation of potential ligand that contribute to metal binding (Tobin et al., 1984).

The contact time between the bacterial cells and the metal solutions is an important factor affecting the metal uptake. Figure 6 shows the maximum uptake for  $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  occurred after 24 h. However, 18 and 48 h was optimum for  $\text{Co}^{2+}$  and  $\text{Pb}^{2+}$  uptake. These results agree with that of Remacle (1990). The uptake of  $\text{Co}^{2+}$  decreased by increasing the contact period between bacteria and metal more than 18 h. The result was also obtained for *B. anthracis* PS2010 by El-Shanshoury et al. (2012). Cell age is considered as an important factor that affects metal accumulation. During the detection of metal uptake with *B. anthracis* PS2010 illustrated in Figure 7, it was found that  $\text{Pb}^{2+}$  was the most highly uptake element while the uptake of  $\text{Cu}^{2+}$  was the lowest for the considered heavy metals. The uptake of the five metals by *B. anthracis* PS2010 was in the following order  $\text{Pb}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+} > \text{Cu}^{2+}$  with different uptake values of  $6.44 \pm 0.63$ ,  $5.22 \pm 0.41$ ,  $4.75 \pm 0.39$ ,  $3.41 \pm 0.47$  and  $2.03 \pm 0.30$   $\text{mg} \cdot \text{g}^{-1}$  dry weight, respectively. This difference in the uptake may be due to the difference in mechanisms by which the bacteria can tolerate the different heavy metals.

The synthesis of Pb nanoparticles by *B. anthracis* PS2010 was detected by examining the cells of *B. anthracis* before and after treatment with 0.4 mM of  $\text{Pb}(\text{NO}_3)_2$ , with TEM. The bacterium was able to synthesize nanostructure particles from Pb (Figure 8), it was clear that these nanoparticles were synthesized extracellularly as a result of lead exposure. The X-ray powder diffraction (XRD) analysis of the dried  $\text{Pb}(\text{NO}_3)_2$ -treated cells indicated the synthesis of lead oxide (PbO) nanoparticles by *B. anthracis* PS2010 (Figure 9). The suggested mechanism for the formation of PbO nanoparticles by *B. anthracis* PS2010 occur in an aerobic condition.

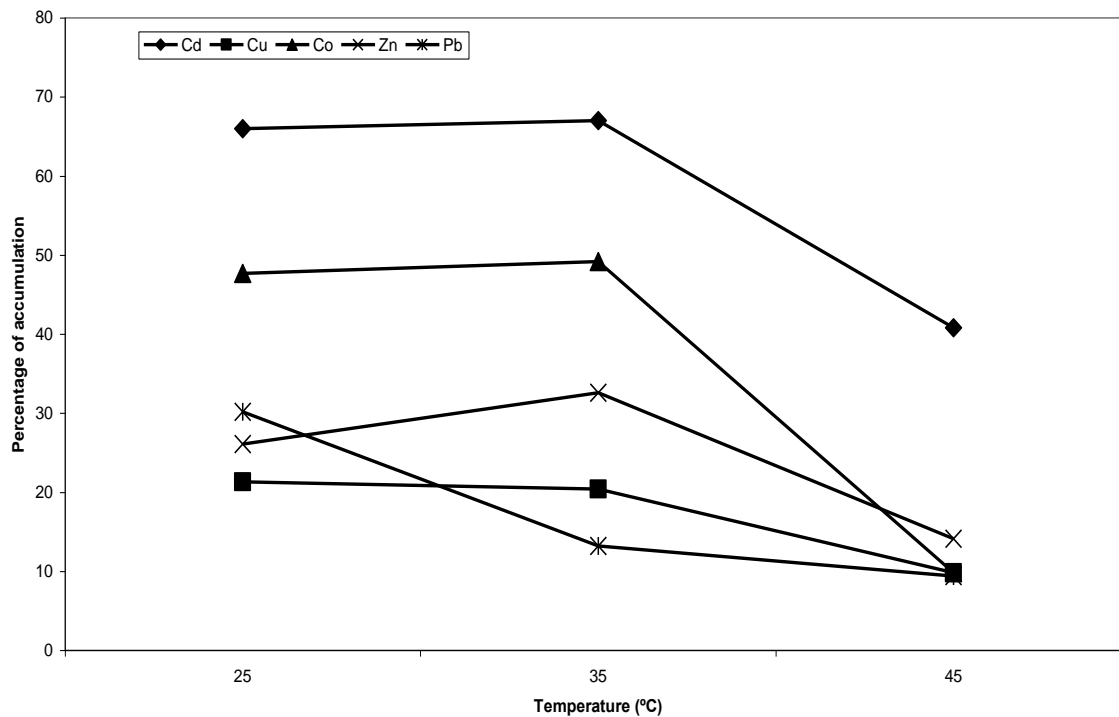


Figure 4. Effect of different temperatures on heavy metal accumulation by *B. anthracis* PS2010.

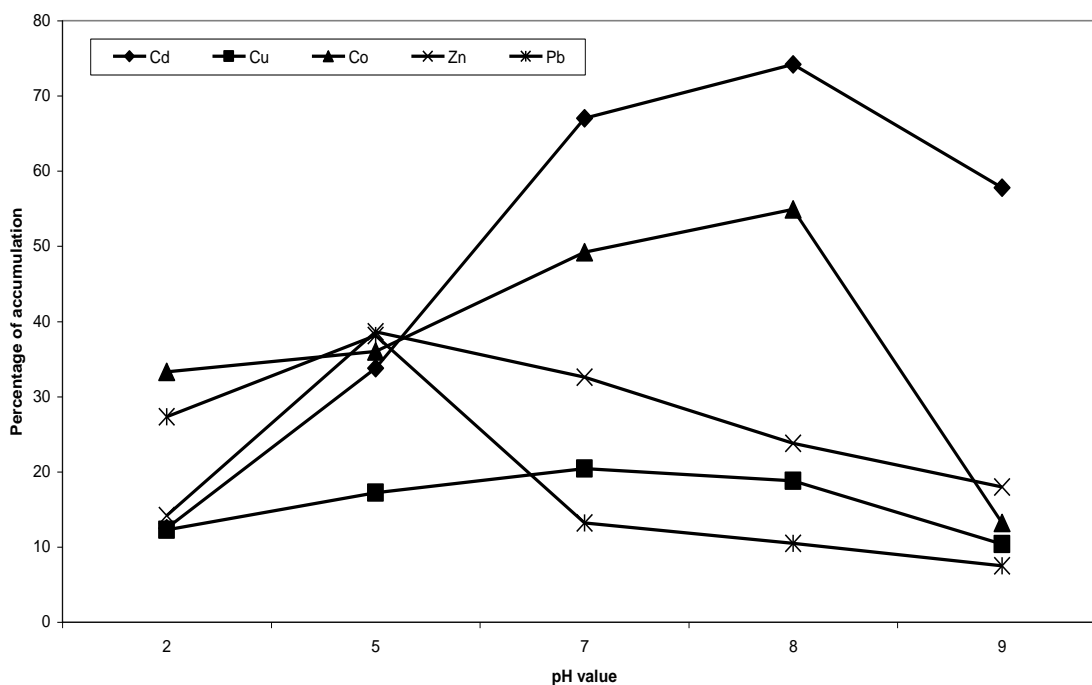


Figure 5. Effect of different pH values on heavy metal accumulation by *B. anthracis* PS2010.

Under these conditions, the production and accumulation of large amounts of sulfide likely occur, which transfer across the membrane into the culture medium and can be used as sulfur source in the formation of PbS nano-

particles. Engels et al. (2000) and Rudzinski et al. (2004) reported that methanethiol under aerobic conditions is converted rapidly to dimethyldisulfide (DMDS) and/or dimethyl-trisulfide which caused precipitation for PbS

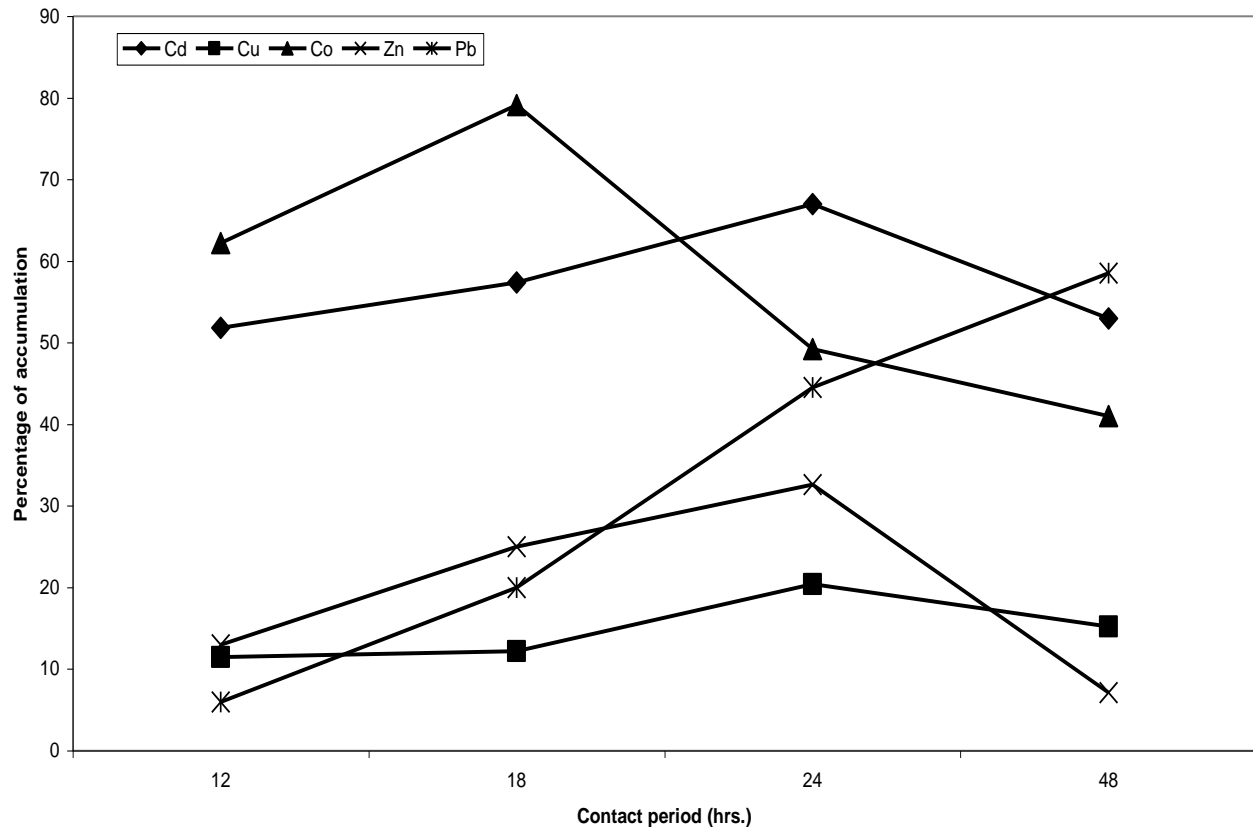


Figure 6. Effect of different contact periods on heavy metal accumulation by *B. anthracis* PS2010.

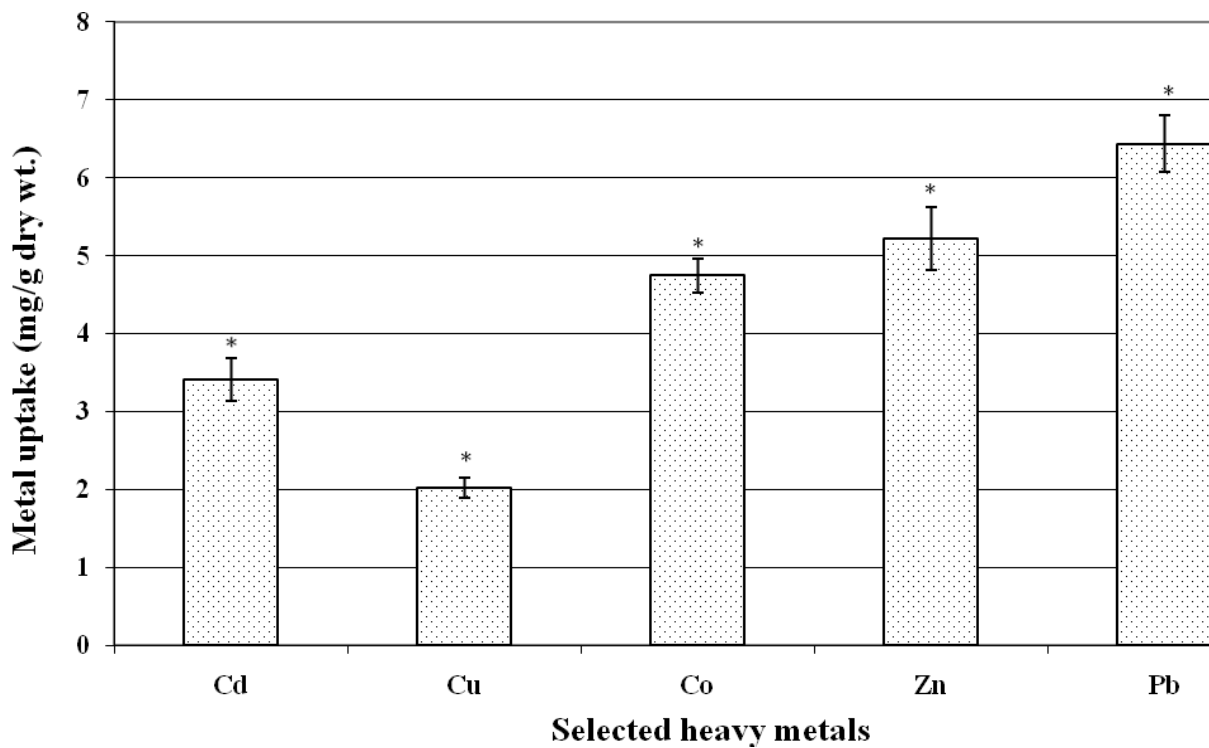
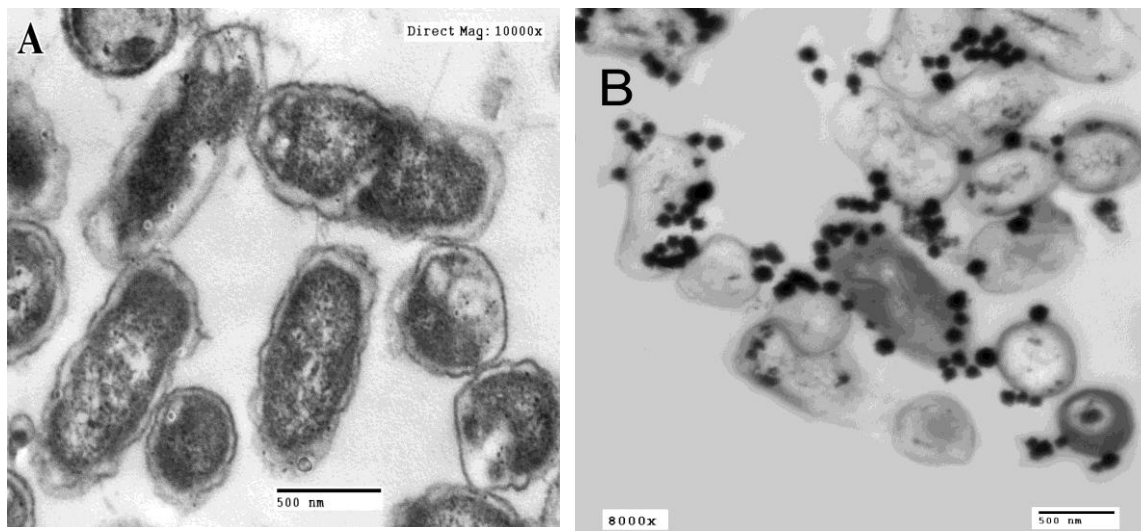
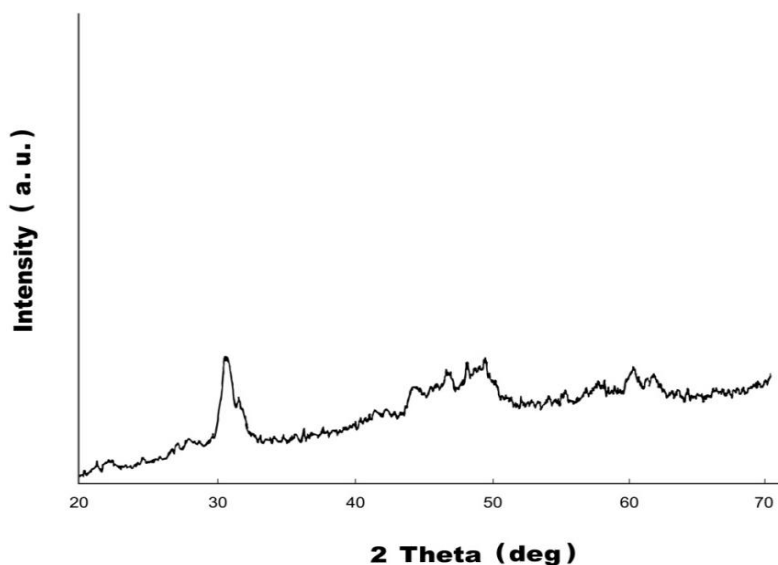


Figure 7. Metals uptake by *B. anthracis* PS2010 under the optimum conditions.



**Figure 8.** Transmission electron micrographs of *B. anthracis* PS2010, A: untreated cell; B: showing extracellular accumulation of Pb nanoparticles.



**Figure 9.** X-ray diffractogram of PbO nanoparticles synthesized by *B. anthracis* PS2010.

nanoparticles. Gong et al. (2007) obtained PbS nanoparticles by *Desulfotomaculum* sp. (strictly anaerobic sulfate-reducing bacteria). This bacterium can utilize sulfate as a terminal electron acceptor in their anaerobic oxidation of organic substrates. As a result, they produce and accumulate large amounts of sulfide which transfer across the membrane into the culture medium and could be used as sulfur source in the formation of PbS nanoparticles. This property of metal particle generation enables the bacteria to work as a living factory and as an inexpensive system to produce metal nanoparticles which have a strong application in the field of material science (Chowdhury et al., 2008). The mechanism of PbS synthe-

sis by *B. anthracis* PS2010 is suggested to be a precipitation of Pb by DMDS off gas produced by the cells from methionine amino acid in the form of PbS nanoparticles (Macaskie et al., 2007).

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## Full Length Research Paper

## Phylotype analysis of *Ralstonia solanacearum* strains causing potato bacterial wilt in Karnataka in India

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Potato is one of the important cash crop of Karnataka (India) farmers and there is a tremendous scope to increase the area and productivity of this crop in the state. However, this crop is badly affected by bacterial wilt disease caused by *Ralstonia solanacearum* especially in Hassan, Chikmagalur and Bengaluru districts. During *Kharif* 2011 season, a total of fifteen bacterial strains were recovered from stem pieces of wilting potato plants collected from potato growing regions of Hassan and Chikmagalur districts in Karnataka. Total genomic DNA was extracted and the identity of all the fifteen strains was confirmed as *R. solanacearum* as expected single 280-bp fragment resulted in all the isolates following polymerase chain reaction (PCR) amplification using the *R. solanacearum* specific universal primer pair 759/760. A phylotype specific multiplex PCR revealed that all the bacterial strains belonged to phylotype I of *R. solanacearum* which correspond to race 1 of the pathogen.

**Key words:** Bacterial wilt, Karnataka, phylotype, potato, *Ralstonia solanacearum*.

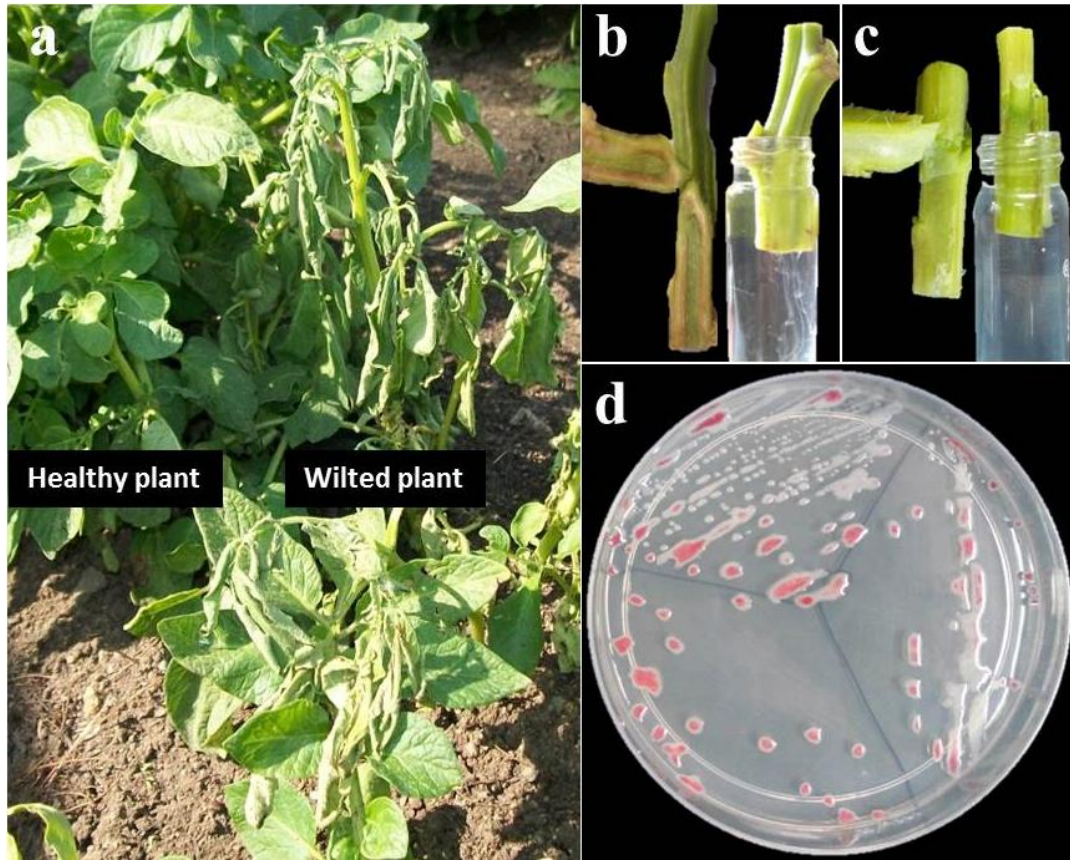
### INTRODUCTION

Potato (*Solanum tuberosum* L.) occupies an area of about 76.8 thousand ha with a total production of 788 thousand tonnes in Karnataka. Among the six potato growing districts, Hassan district alone contributes more than 50% of area and potato production in Karnataka. It is one of the important cash crop of Karnataka farmers, and there is a tremendous scope to increase the area and productivity of this crop in the state (Basvaraja et al., 2009). However, potato production is adversely affected

by bacterial wilt disease especially in Hassan, Chikmagalur and Bengaluru districts (Anonymous, 2011).

Bacterial wilt (brown rot) incited by *Ralstonia solanacearum* (Yabuuchi et al., 1995) is a major constraint on potato production worldwide and in many potato growing regions of India (Elphinstone, 2005; Sagar et al., 2014). Historically, *R. solanacearum* has been divided into five races related to the ability to wilt members of the family *Solanaceae* (race 1), banana

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**Figure 1.** Symptoms of bacterial wilt of potato caused by *R. solanacearum* (a); brown discoloration of vascular tissues in stem and bacterial streaming in clear water from stem of infected plant (b) in comparison with healthy plant stem (c) and typical *R. solanacearum* colonies on TZC agar medium (d).

(race 2), potato and tomato in temperate conditions (race 3), ginger (race 4) and mulberry (race 5) and six biovars related to the ability to metabolize three sugar alcohols and three disaccharides (He et al., 1983). Based on this classification, potatoes are known to be affected by two races of *R. solanacearum*, that is, race 3 inducing wilt of potatoes under cool temperate conditions and race 1 damage potato crops under tropical and subtropical conditions (Martin and French, 1985).

Recently, a new phylogenetic classification system was proposed by Fegan and Prior (2005), consisting of four phylotypes, each further divided into sequevars. By using the *R. solanacearum* species-specific primers 759/760 in combination with phylotype-specific primers (Nmult:21:1F, Nmult:21:2F, Nmult:23:AF, Nmult:22:InF, and Nmult:22:RR), species and phylotype affiliation can be simultaneously identified in a single PCR assay, called the phylotype-specific multiplex PCR (Pmx-PCR). Depending on Pmx-PCR product patterns, strains of *R. solanacearum* can be grouped into the four phylotypes. Phylotype I (Asiatic origin) is characterised by production of 280 and 144 bp amplicons. Phylotype II strains (American origin) produce 280 and 372 bp amplicons. Phylotype III (mainly

from Africa and nearby islands such as Reunion and Madagascar) produce 280 and 91 bp amplicons. Phylotype IV strains (from Indonesia, Japan, and Australia) produce 280 and 213 bp amplicons. The phylotyping scheme adds valuable information about the geographical origin and in some cases the pathogenicity of strains. Therefore, the present investigation was aimed to use phylotyping scheme to determine the phylotypes of the *R. solanacearum* strains causing potato bacterial wilt in Hassan and Chikmagalur districts of Karnataka state in India.

## MATERIALS AND METHODS

### Bacterial strains, media and growth condition

A total of 15 strains of *R. solanacearum* were isolated from bacterial wilt affected potato plants (Figure 1a) collected from potato growing regions of Hassan and Chikmagalur districts of Karnataka in India during *Kharif* 2011 season. Stem pieces (5-6 cm long) of wilted potato plants were collected from each field, washed thoroughly, air dried and brought to the laboratory for further studies. The samples were then surface disinfected with 70% ethanol, peeled, sub sampled and macerated in sterile distilled water. Macerates were streaked on Kelman's triphenyltetrazolium chloride (TZC) agar medium

**Table 1.** List of primers used for multiplex PCR.

Primer Name	Primer sequence	Expected band size (bp)	Remark
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	Phylotype I (Asiaticum)
Nmult:21:2F	5'-AAGTTA TGGACGGTGAAGTC-3'	372	Phylotype II (Americanum)
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3	213	Phylotype IV (Tropical)
Nmult:23:AF	5'-ATTACGAGAGCAATC GAAAGATT-3'	91	Phylotype III (African)
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3		Amorce reverse unique
759R	5'-GTCGCCGTCAACTCACTTTCC-3'		Universal <i>R. solanacearum</i>
760F	5'-GTCGCCGTGAGCAATGCGGAATCG-3'	280	specific primers

(Kelman, 1954) (Peptone, 10 g; glucose, 2.5 g; Casamino acid, 1 g; agar, 18 g; TZC, 50 mg L<sup>-1</sup>; pH 7.0-7.1). Plates were incubated at 28±2°C for 48 to 72 h. Bacterial colonies developing the typical irregular mucoid colonies were again streaked onto fresh TZC medium for further purification. Well separated typical wild type *R. solanacearum* colonies were further transferred to medium modified by exclusion of TZC for multiplication of inoculum. Two loops of bacterial culture were then transferred in 2 ml of double distilled sterile water and the cultures were stored at 20±2°C.

#### DNA extraction from bacterial strains

Total genomic DNA was extracted as described by Chen and Kuo (1993). A well separated bacterial colony on TZC agar was used to inoculate 1.5 ml of CPG broth (Peptone, 10 g; glucose, 2.5 g; Casamino acid, 1 g; distilled water 1 litre; pH 7.0-7.1) in 2.0 ml Eppendorf tubes. The cultures were grown at 28±2°C for 48 h with vigorous shaking. Each culture (1.5 ml) was harvested by centrifugation for 3 min at 13,000 xg. The cell pellet was re-suspended and lysed in 300 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS, 20 µg RNase A) by vigorous pipetting and incubated for 30 min at 37°C. To remove most proteins and cell debris, 100 µl of 5 M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 13,000 xg for 10 min at 4°C. After transferring the clear supernatant into a new vial, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 13,000 xg for 3 min, the extracted supernatant was transferred to another vial and the DNA was precipitated with 100% ethanol, washed twice with 70% ethanol, dried in speed-vacuum, and re-dissolved in 50 µl of TE buffer.

#### Phylotype analysis

Phylotype identification of each strain was done as described (Fegan and Prior, 2005; Prior and Fegan, 2005). Phylotype specific multiplex PCR (Pmx-PCR) was carried out in 25 µl final volume of reaction mixture, containing 1xTaq Master Mix (PCR buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris-HCl and 1.25U of Taq DNA polymerase.), 6 pmoles of the primers Nmult: 21: 1F, Nmult:21:2F, Nmult:22:InF, 18 pmoles of the primer Nmult:23: AF and 4 pmoles of the primers 759 and 760 (Opina et al., 1997) (Table 1). The following cycling programme was used in a thermal cycler (Gen-AmpR PCR System 9700 of M/S Applied Biosystem): 96°C for 5 min and then cycled through 30 cycles of 94°C for 15 s, 59°C for 30 s and 72°C for 30 s, followed by a final extension period of 10 min at 72°C. A 5 µl aliquot of each amplified PCR product was subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide and bands were visualized on a UV-transilluminator. This Pmx-PCR amplifies the 280-bp "universal" *R. solanacearum*

specific reference band plus following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372- bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains; and a 213-bp amplicon from phylotype IV strains.

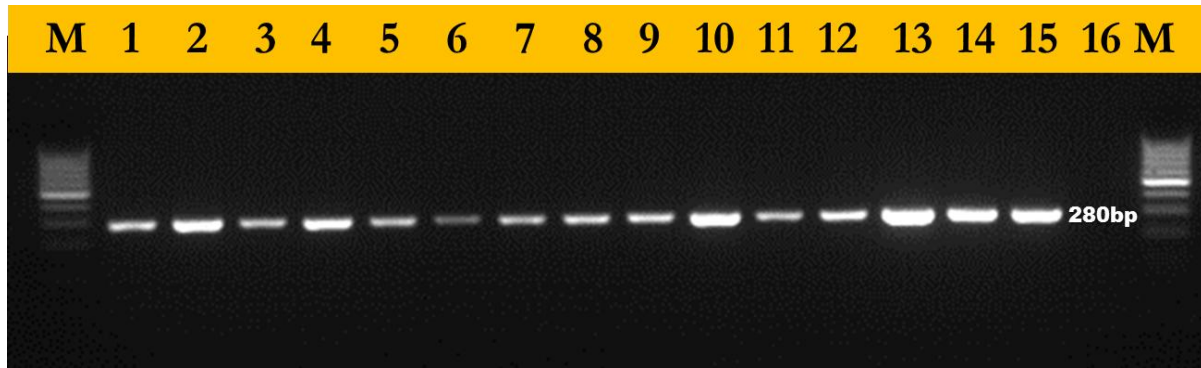
## RESULTS AND DISCUSSION

### Bacterial strain collection and their identification

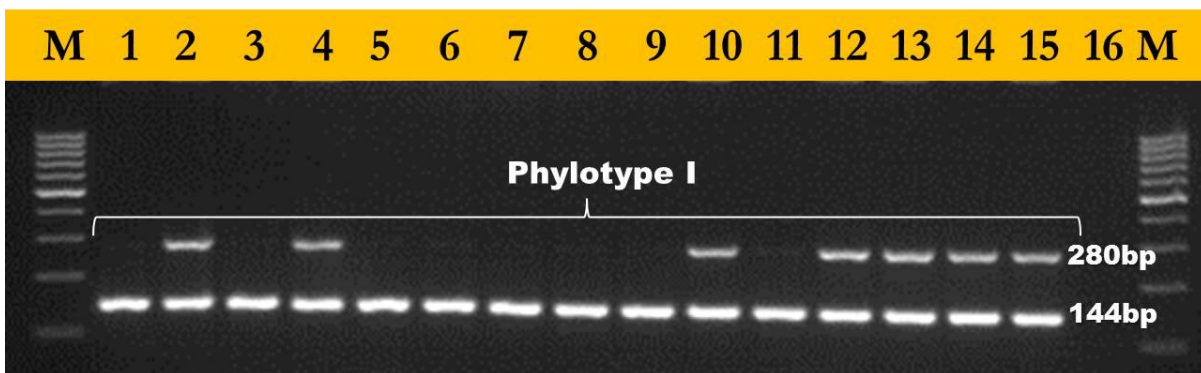
During the present studies, bacterial wilt infected potato stems were collected in *Kharif* 2011 season from wilt affected areas of Hassan and Chikmagalur districts of Karnataka state. A total of 15 bacterial strains were recovered from wilt affected potato stems. On Kelman's (1954) TZC agar medium, these strains yielded typical virulent type colonies, which were cream coloured, irregularly shaped, highly fluidal with pink pigmentation in the centre (Figure 1d). These characters were consistent with *R. solanacearum* as described by Kelman (1954) on TZC agar medium. Total genomic DNA of all the strains was extracted and subjected to PCR amplification using the *R. solanacearum* specific universal primer pair 759/760. An expected single 280-bp fragment (Opina et al., 1997) amplified in all the strains (Figure 2), which further confirmed the identity of these strains as *R. solanacearum*.

### Phylotype identification

Phylotype specific multiplex PCR revealed that all the fifteen strains from Karnataka belonged to phylotype I as a 144-bp amplicon was observed in all the strains when Pmx-PCR products of these strains were subjected to electrophoresis on 2% agarose gel (Figure 3). Phylotype I strains causing bacterial wilt of potato includes *R. solanacearum* strains traditionally classified as biovar 3, 4 and 5; are primarily isolated in Asia (Fegan and Prior, 2005) and correspond to race 1 of the pathogen. Also, *R. solanacearum* strains which cluster into phylotype I encompass a majority of lowland (tropical) strains with a wide host range (Cellier and Prior, 2010). Shekhawat et al. (1978) have also reported race 1 biovar 3 of *R. solanacearum* as cause of bacterial wilt of potato in plains and plateau region of India and our results are in conformity with this.



**Figure 2.** Single 280-bp fragment resulting from PCR amplification using *R. solanacearum* specific universal primer pair 759/760 (Lane M = 1 kb ladder, lane 1-15 = strains of *R. solanacearum*, lane 16 = control).



**Figure 3.** Phylotype specific multiplex PCR of 15 isolates of *R. solanacearum* from Karnataka (Lane M = 1 kb ladder, lane 1-15 = strains of *R. solanacearum*, lane 16 = control).

Knowledge of local pathogen diversity is a key prerequisite for successful integrated disease management programme. Varieties known to be resistant to strains of one phylotype of *R. solanacearum* may become susceptible to strains of other phylotype of *R. solanacearum* (Suga et al., 2013). In Hassan and Chikmagalur districts of Karnataka state, bacterial wilt of potato is caused by strains of phylotype I of *R. solanacearum* (this study). These phylotype I strains are different from those reported recently by Sagar et al. (2014) as cause of bacterial wilt of potato in Madhya Pradesh (phylotype II strains), West Bengal (phylotype II strains) and in Meghalaya (phylotype II and IV strains) states in India. Race 3 biovar 2 (phylotype II strains) is primarily pathogenic to potato and persist only under cool humid conditions whereas, race 1 (phylotype I strains) have wide host range and survive in warm areas (Shekhawat et al., 1992). While the occurrence of phylotype II strains in warm sub-tropical climatic conditions in India is attributed to latently infected tubers (Sagar et al., 2013), phylotype I strains are anticipated to cause bacterial wilt of potato in low land (tropical) areas (Martin and French, 1985; Shekhawat et al., 1992).

## Conclusion

Bacterial wilt disease of potato is caused by strains of phylotype I of *R. solanacearum* in Karnataka which differ from strains of *R. solanacearum* in Madhya Pradesh, West Bengal and Meghalaya states in India. The occurrence of Phylotype I of *R. solanacearum* in Karnataka may be attributed to the climatic condition and cropping pattern in the state. Particularly in Hassan and Chikmagalur districts, potato crop is grown in rotation with other solanaceous vegetables like tomato, brinjal, etc. This helps in perpetuation of this particular phylotype which also has wide host range and survive better in tropical climate. Phylotype I strains correspond to race 1 of the pathogen and cause potato bacterial wilt in low land (tropical) areas.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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## Full Length Research Paper

# Molecular typing and cytotoxicity testing of *Staphylococcus aureus* isolated from milk, meat and clinical sources

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***Staphylococcus aureus* is one of the main causes of foodborne diseases and a leading cause of nosocomial infections worldwide. Eleven (11) isolates from milk; 34 from meat and 23 of clinical source were subjected to phenotypic and biochemical characterizations for confirmation and further analysis for the presence of virulent genes, clonal relatedness and cytotoxicity profile. All isolates were positive for catalase production; 3, 11, and 7 isolates from milk, meat and clinical sources were coagulase positive. Polymerase chain reaction technique was used for the detection of 16S rRNA, clumping factor (*clfA*) and methicillin resistance (*mecA*) virulent genes. All isolates were positive for 16S rRNA; 100 and 45% of clinical; 73 and 64% of milk and none of the meat isolates were positive for *clfA* and *mecA* genes, respectively. Eight *Sma*I-based pulse-field gel electrophoresis (PFGE) clusters were identified at 80% similarity. Cytotoxic potential of the strains showed that 27.8, 24.1 and 22.2% of the strains tested had high, medium and low lactate dehydrogenase (LDH) release, respectively. These findings clearly demonstrate the presence of *S. aureus* in food and also in clinical infections, thus it should be a public health concern because even when virulent genes were not amplified in some strains, there may still be cytotoxicity effects.**

**Key words:** *Staphylococcus aureus*, pulse-field gel electrophoresis (PFGE), lactate dehydrogenase (LDH), cytotoxicity profile, molecular typing.

## INTRODUCTION

*Staphylococcus* species are found normally in hair and skin of humans and animals, and according to many researchers, they are currently the leading cause of nosocomial infections worldwide (Lowy, 2003; Michelim et al., 2005; Bayles et al., 1998). *Staphylococci*, espe-

cially *Staphylococcus aureus*, are opportunistic pathogens if infection occurs in the susceptible individuals (Ghebremedhin et al., 2007).

Infections associated with *S. aureus* are extremely common, ranging from furuncles to foodborne intoxication

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and life threatening disease; therefore, the pathogen has potential to cause increased morbidity and mortality (Bayles et al., 1998). A recent study shows that almost 50% of meat in the grocery stores in the United States is contaminated with *S. aureus* (Waters et al., 2011); it is also the leading cause of intra-mammary infection in dairy animals. Furthermore, comprehensive epidemiological analysis has shown that *Staphylococcus* spp. was involved in several cases of outbreaks in United States (Ajayi et al., 2011).

According to reviews in Food Science and Food Safety (2006), there are antimicrobial usages at several stages in the food system, from the environment into the kitchen table, which creates an increasing spread of multi-drug resistant *S. aureus* in food worldwide and is a threat to food safety creating a public health concern (Xie et al., 2011). Because of the spread of multi-drug resistant pathogens in foods, the *Staphylococcus* spp. was subjected to resisto typing, applying 14 classes of antibiotics in order to establish the antimicrobial resistance profile of the isolates. It was found that majority of the isolates were resistant to 13 out of the 14 antibiotics tested (Ajayi et al., 2009).

Additionally the study on virulence genes characterization of *Staphylococcus* spp. isolated from food and clinical sources is important to estimate the prevalence of infections. Pulse-field gel electrophoresis (PFGE) is known to be a highly discriminatory technique, often used for characterizing genetic diversity of isolates and to research on outbreak sources (Xie et al., 2011). Furthermore, PFGE is particularly effective for molecular typing of methicillin resistant *S. aureus* (MRSA) (van Belkum et al., 1998).

The persistence of staphylococci in its hosts, despite the initiation of humoral and mucosal antibodies, continues to be an important issue for health of both humans and animals (Bayles et al., 1998). Furthermore, bacteria such as *Listeria monocytogenes*, *Salmonella* spp. and *Shigella* spp. are known to evade humoral immunity and then become internalized in host cells (Bayles et al., 1998). Although *S. aureus* is generally not considered to be an intracellular pathogen, it can be internalized, inducing apoptosis (cell death) in various cell types including epithelial cells, endothelial cells, keratinocytes, osteoblasts, lymphocytes and macrophages (Bayles et al., 1998; Wesson et al., 2000; Menzies and Kourteva, 1998; Nuzzo et al., 2000; Tucker et al., 2000; Jonas et al., 1994). The human INT-407 cell line is used in pathogenic enterobacterium studies, and to study cytotoxicity potential of foodborne pathogen *Escherichia coli*, (Maldonado et al., 2005), *Salmonella enterica* serovar Typhi (Santos et al., 2001) and *Klebsiella pneumoniae*.

Lactate dehydrogenase (LDH) is a low molecular weight enzyme, easily released from cell as a result of minor disturbances to the cell membrane integrity (Bhunja, 2008), and the release of LDH is one of the

most widely used and accepted methods for measuring cellular death/lysis (Moran and Schnellmann, 1996). One of the advantages of this assay is that it can detect minimal membrane damage. Therefore, because of the prevalence in nature and the role of *S. aureus* in foodborne diseases and nosocomial infections, the focus of this study are to examine the virulence genes using multiplex PCR and the genetic relatedness of the strains using PFGE. Secondly, we aimed to determine the cytotoxic potential of strains isolated from milk, meat and clinical sources.

## MATERIALS AND METHODS

### Isolation of *S. aureus* from clinical, rabbit meat and milk samples

The clinical samples were donated from a local hospital and the milk samples were collected weekly from a local dairy (Huntsville, AL) and processed in the Food Microbiology Laboratory at Alabama A&M University. Isolation of *S. aureus* was conducted according to the procedure described by the FDA-Bacteriological Analytical Manual (FDA, 1998). For initial isolation of *Staphylococcus* in meat (Rabbit), one hind leg from each whole carcass was placed in a sterile Stomacher bag with 0.1% (w/v) peptone (Oxoid, Lenexa, KS) water and vigorously shaken for 2 min and further dilutions were prepared. Approximately 0.1 ml of each dilution was plated onto Baird-Parker agar (BPA) supplemented with egg yolk tellurite (Merck, Gibbstown, NJ) and incubated at 37°C for 24 h. For isolation of *S. aureus* from milk or clinical samples, a 0.1 ml of both undiluted and 1:10 serially diluted sample of each was plated and incubated as earlier described.

Plates containing colonies with typical growth morphology of *S. aureus* were selected, isolated and sub-cultured in trypticase soy broth (TSB). Staphylococcal isolates were identified by colony morphology, catalase, Gram-stain, Staphylococcal coagulase reaction and clumping factor (agglutination) (Food and Drug Administration, 1992). Identification was confirmed with API kits, either API STAPH or ID 32 STAPH (bioMerieux NA, Durham, NC).

### Detection of virulence genes using multiplex polymerase chain reaction (PCR)

Isolation of bacterial DNA from overnight cultures was performed according to Karahan and Centinkaya (2006). PCR assays were performed in an Eppendorf Mastercycler (epGradient S model Thermal Cycler, NY, USA). PCR protocol and primers corresponding to *S. aureus* regions to 16S rRNA portion, clumping factor (*clfA*) and methicillin (*mecA*) genes of *S. aureus* as described by (Mason et al., 2001) were used. The primers used in this study were as follow: *Staphylococcus* specific 16S rRNA gene (791bp), 16S-F (5'-CCTATAAGACTGGGATAACTTCGGG 3') and 16S-R (5'-CTTTGAGTTTCAACCTTGCGGTCG 3'); clumping factor gene (638bp), *clfA*-F (5'-GCAAAATCCAGCACAAACAGGAAACGA-3') and *clfA*-R (5'-CTTGATCTCCAGCCATAATTGGTGG-3'); and methicillin resistant gene (499bp), *mecA*-F (5'-TCCAGGAATGCAGAAAGACCAAAGC-3') and *mecA*-R (5'-GACACGATAGCCATCTTCATGTTGG-3') Invitrogen (Carlsbad, California).

*S. aureus* ATCC 700698, and *E. coli* which served as the positive and negative controls respectively were included in the PCR reactions. About 5 µL aliquot of PCR products was mixed with 3 µL loading dye and with molecular weight marker of 100-bp, were ana-

lyzed by electrophoresis on a 1% agarose gel at 70V for 1 h. Gels were stained in ethidium bromide, viewed by ultra-violet (UV) trans-illumination and photographed.

### Pulsed PFGE

Only *Staphylococcus* isolates that tested positive for either *clfA* or *mecA* virulence genes were subjected to PFGE for research on clonal relatedness.

### Preparation of DNA plugs

Chromosomal DNA was prepared as described by Reed et al. (2007). Briefly, 5 µL of lysostaphin enzyme was added to cell suspension equivalent to 3-4 x 10<sup>9</sup> CFU/mL, plus the addition of 250 µL of 60°C 1.8% SeaPlaque agarose (FMC Bio-product). Suspension was mixed well and 250 µL was immediately transferred into the plug molds. Plugs were allowed to solidify for 10 min at 4°C, washed and DNA plugs were cut and placed in an eppendorf tube containing 125 µL of 1X *Sma*I restriction buffer. Then, 30 U of *Sma*I restriction enzyme (Roche Diagnostics, Mannheim, Germany) was added to each tube, mixed gently and incubated for 2 h at 25°C. The digested DNA plugs were resolved on a 1% gel on a Chef Mapper (BioRad, Hercules, CA, USA), PFGE equipment with CHEF MAPPER parameters set as follows: 5 s initial switch time; 40 s final switch time; 20 h duration of run; voltage 200 V or 6 V/cm gradient; angle of 120° and chiller at 14°C. All gels were stained with ethidium bromide, documented and analyzed with a Foto/analyst Luminary FX Electrophoresis Documentation and Analysis System (Fotodyne Inc. Rochester, NY). Similarity indices were determined using the Dice coefficient (Dice, 1945) and the distances between clusters were calculated using the unweighted pair group method with arithmetic averages (UPGMA). Data analysis was performed using SPSS version 12.0.1. (SPSS Inc. Chicago, IL). Interpretations of PFGE patterns were performed according to Tenover et al. (1995).

### Preparation of mammalian cell culture

The Human jejunal (INT-407) cell line was obtained from American Type Culture Collection (ATCC CCL-6), cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (D<sub>10</sub>F). Stock INT-407 cultures were maintained in a 37°C incubator with 7% CO<sub>2</sub> for 3 weeks to ensure a uniformed monolayer of culture. Confluent monolayer used for cytotoxicity assay was trypsinized, re-suspended in 24-well plates with fresh medium and incubated for 48 h.

### Preparation of bacterial toxin

#### Bacterial cultures

Stock *Staphylococci* cultures isolated from food and clinical sources were maintained in Trypticase Soy Broth (TSB) with 20% glycerol and stored at -80°C. Prior to use, each strain was sub-cultured in 5 mL TSB and incubated overnight at 37°C.

#### Toxin preparation

Toxin was prepared as described by Maldonado et al. (2005). Briefly, a 1.5 mL aliquot of overnight bacterial culture was centrifuged

at 10,000 rpm for 3 min and cell-free supernatants were stored in sterile tubes. Cell pellets were re-suspended in 75 µL of polymyxin B sulfate solution (2 mg/mL of PBS), incubated for 30 min and centrifuged. Following centrifugation, the supernatants were collected, combined with the original cell-free supernatants and filtered through 0.45 µm disc filters (Corning, NY). The filtrates were used immediately or stored at 4°C for up to two days.

### Lactate dehydrogenase (LDH) assay

The cytotoxic effect of *Staphylococcus* on human cell lines was assessed, by measuring lactate dehydrogenase (LDH) released according to Roberts et al. (2001), with slight modification. Briefly, confluent INT-407 cells transferred to 24 well plates were used upon formation of monolayer. Each well was washed three times in 900 µL serum free (D0F) DMEM, then the low (PBS), high (1% Triton-X) controls and samples were prepared in triplicates in a 24 well plates as follows: (900 µL D<sub>0</sub>F DMEM and 100 µL of controls or bacterial toxin filtrates), and incubated for 16 h at 37°C with 7% CO<sub>2</sub>. After incubation, a 100 µL aliquot of supernatant from each bacterial isolate was dispersed into triplicate wells of a 96 well plates. Lactate dehydrogenase substrate was prepared according to the manufacturer's guidelines (Roche Applied Science, Indianapolis, IN) and 100 µL was dispensed into each well. Plates were incubated at room temperature and the absorbance was measured at a dual wavelength of 490 and 655 nm after 5 min and subsequently every 3 min for 15 min by a plate reader (Synergy HT, Vermont, USA). Percentage of cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100$$

### Statistical analysis

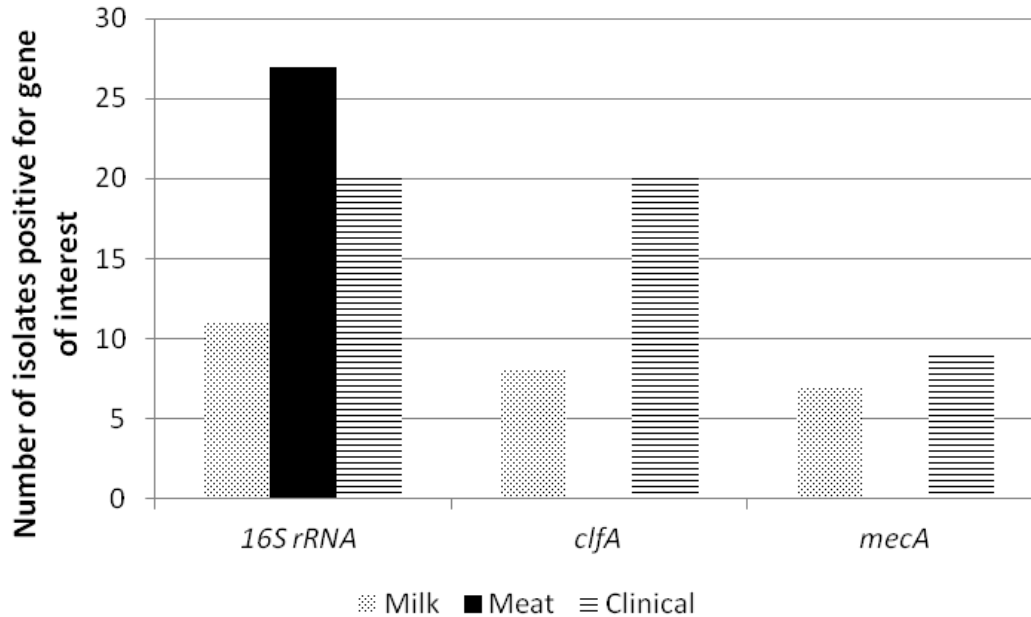
Calculated percent cytotoxicity data were analyzed using Statistical Package for the Social Sciences (SPSS 16). Averages of the triplicates plus Standard deviation were reported. Tukey's studentized test was used for the separation of means and P<0.05 was taken as statistically significant association.

## RESULTS

### Multiplex PCR: 16S rRNA; (*clfA*) and (*mecA*) genes

Multiplex PCR analysis was performed on 61 samples of presumptive *Staphylococcus* isolates; 11 from milk; 27 from meat and 23 from human infections (clinical) and *S. aureus* ATCC 700698, and *E. coli* which served as the positive and negative controls respectively were tested. PCR amplifications yielded the fragments of the expected sizes. All *Staphylococcus* isolates were confirmed positive for 16S rRNA, 28 isolates for *clfA* and 16 strains were positive for *mecA* gene (Figure 1).

Clumping factor (*clfA*) gene was found in 20 clinical and eight milk isolates. None of the strains isolated from rabbit meat were positive for clumping factor gene. The *mecA* gene which resulted in a 499 bp fragment was found in 9 clinical and 7 milk isolates, while none of the meat isolates showed positive *mecA* gene (Figure 1). The



**Figure 1.** Detection of 16S rRNA, clumping factor (*clfA*) and methicillin resistant (*mecA*) genes from *S. aureus* in milk, meat and clinical isolates.

complete results of the presence of 16S rRNA, *clfA* and *mecA* virulent genes for each strain are summarized in Table 1.

### Clonal relatedness in *Staphylococcus*

To investigate the clonal relationship, a total of 33 *S. aureus* strains (9 isolates from milk and 24 from clinical sources) were subjected to *Sma*I macro restriction fragment analysis of their chromosomal DNA by PFGE, including 2 ATCC (8325-4 and 700698) strains of *S. aureus* isolates. The PFGE profiles of the clinical isolates were compared with the PFGE profile of the milk isolates.

As reported by Tenover et al. (1995), isolates are designated genetically indistinguishable if the restriction patterns have the same number of bands and the bands appear to be of similar size. Figure 2A is representative of the PFGE gels for clinical strains, two strains in lanes 3 and 4 had PFGE banding patterns that were indistinguishable.

The other clinical strains were closely related, since there were two to three differences in bands between the observed patterns, possibly due to point mutations (insertions or deletions). The *S. aureus* strains from milk are shown in Figure 2B. Lanes 1, 7 and 15 correspond to ATCC 700698 and lanes 6, 8 and 13 to one milk sample that appeared to be genetically indistinguishable.

Of the 33 isolates typed by *Sma*I-based PFGE, each of the two ATCC strains had distinct PFGE patterns different from the strains isolated from milk or clinical sources. The horizontal bar above the dendrogram indicates the percentage of genetic relatedness among the

different strains. Each cluster had varying number of strains.

### Cytotoxicity profile

A total of 57 *Staphylococcus* isolates were evaluated for their cytotoxicity potential, based on the release of lactate dehydrogenase from jejunal (INT-407) cells, and were compared with the positive control, Triton-X 100 (1%) and PBS and the negative control. Cytotoxicity assay were performed in triplicate. The LDH release values for Triton-X 100 ranged from 1.459 to 1.47 and the negative control ranged from 0.334 to 0.338. The results of LDH values (averages of absorbance  $\pm$  standard deviations) and calculated percent cytotoxicity for each isolate tested are listed in Table 1.

The percent of cytotoxicity were grouped as negative (<1%); low (1 to 20%); medium (21 to 49%) and high (>50%) categories arbitrarily according to Maldonado et al. (2005). Four (36%) from milk, 9 (33%) from meat and 1 (5.5%) from clinical isolates were in the negative category (Figure 4).

Three (27%), 4 (14%) and 5 (31%) from milk, meat and clinical respectively were in the low category; 2 (18%) 5 (18.5%) and 6 (33%) isolates from milk, meat and clinical were in the medium category while 1 (9%) from milk, 9 (32%) from meat and 4 (22%) clinical isolates were in the high category.

The strains isolated from milk had LDH had range from -24 to 80%; the strains from meat ranged from -27 to 194%; while the clinical strains LDH ranged from -17 to 113%. There was a statistically significant difference ( $p < 0.05$ ) in LDH release between the clinical and milk

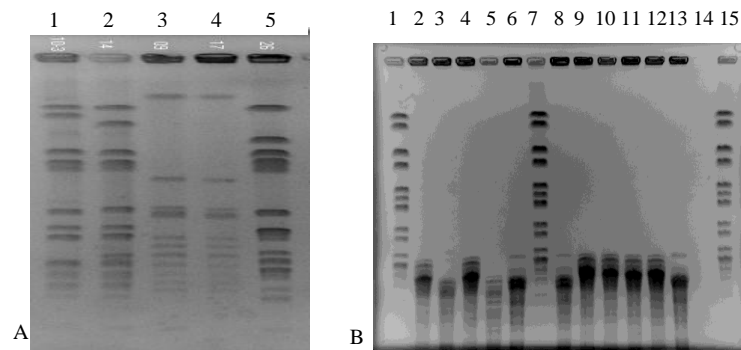
**Table 1.** Cytotoxicity Profile of *Staphylococcus aureus* isolated from milk, meat and clinical sources.

Isolate	Source	Coagulase	Virulence genes present <sup>a</sup>			Cytotoxicity <sup>b</sup>	
			16S rRNA	<i>clfA</i>	<i>mecA</i>	LDH ( <i>A</i> <sub>490/655</sub> )	%
Low control	PBS					0.347	
High control	Triton X					1.464	
M 11	Milk	NT <sup>c</sup>				0.302 ± 0.02 <sup>xy</sup>	- 4
M 3-5	Milk	+	+	+	+	0.413 ± 0.021 <sup>wx</sup>	6
M 9	Milk	-	+	+	+	0.144 ± 0.004 <sup>y</sup>	- 18
M 8	Milk	-	+	+	+	0.481 ± 0.046 <sup>p</sup>	12
M 4-3	Milk	-	+	+	+	0.155 ± 0.004 <sup>y</sup>	- 17
M 4-1	Milk	-	+	+	+	0.872 ± 0.044 <sup>h</sup>	47
M 3-1	Milk	NT	+	+	-	NT	
M 3-2	Milk	+	+	-	-	0.077 ± 0.004 <sup>z</sup>	- 24
M 3-3	Milk	-	+	+	+	0.374 ± 0.033 <sup>xy</sup>	2
M 3-4	Milk	+	+	+	+	0.719 ± 0.007 <sup>lm</sup>	33
M 4-4	Milk	NT				1.238 ± 0.074 <sup>e</sup>	80
PC8	Meat	-	+	-	-	2.509 ± 0.107 <sup>a</sup>	194
PC5-12	Meat	-	+	-	-	0.749 ± 0.030 <sup>kl</sup>	36
PC5	Meat	-	+	-	-	0.717 ± 0.338 <sup>lm</sup>	33
PC7-14	Meat	-	+	-	-	1.729 ± 0.051 <sup>b</sup>	123
PC7	Meat	+	+	-	-	0.423 ± 0.009 <sup>wx</sup>	7
PV1-16	Meat	NT	+	-	-	0.919 ± 0.190 <sup>fg</sup>	51
PV 2-17	Meat	-	+	-	-	1.659 ± 0.228 <sup>bc</sup>	117
PV 4-18	Meat	+	+	-	-	0.217 ± 0.008 <sup>xy</sup>	-12
PV 5-2	Meat	+	+	-	-	0.080 ± 0.001 <sup>z</sup>	-23
PV 5	Meat	+	+	-	-	0.717 ± 0.338 <sup>lm</sup>	33
PV 6	Meat	-	+	-	-	0.164 ± 0.017 <sup>yz</sup>	-16
PV 7-21	Meat	NT				0.072 ± 0.002 <sup>z</sup>	-24
PV 8-22	Meat	-	+	-	-	0.230 ± 0.011 <sup>xy</sup>	-10
PR10	Meat	+	+	-	-	0.181 ± 0.025 <sup>yz</sup>	-14
PR11	Meat	+	+	-	-	0.441 ± 0.043 <sup>uv</sup>	8
PR 2	Meat	+	+	-	-	0.761 ± 0.107 <sup>jk</sup>	37
PR 3R3	Meat	-	+	-	-	0.159 ± 0.007 <sup>yz</sup>	-17
PR 5-3	Meat	+	+	-	-	0.069 ± 0.005 <sup>z</sup>	-24
PR 5	Meat	-	+	-	-	0.410 ± 0.008 <sup>wx</sup>	6
PR 7-4	Meat	+	+	-	-	0.912 ± 0.016 <sup>fg</sup>	51
PR 9-6	Meat	+	+	-	-	1.488 ± 0.042 <sup>d</sup>	102
PC10	Meat	NT	+	-	-	0.383 ± 0.063 <sup>x</sup>	3
PC13	Meat	NT	+	-	-	0.907 ± 0.053 <sup>fg</sup>	50
PC3-16	Meat	NT	NT			0.582 ± 0.225 <sup>qr</sup>	21
PC4-11	Meat	-	+	-	-	1.303 ± 0.101 <sup>de</sup>	86
PR 9 (6-1)	Meat	+	+	-	-	1.805 ± 0.228 <sup>b</sup>	130
PR9	Meat	-	+	-	-	0.413 ± 0.003 <sup>q</sup>	-27
C10-12	Clinical	-	+	+	+	0.599 ± 0.086 <sup>q</sup>	23
C1	Clinical	-	+	+	-	0.687 ± 0.061 <sup>mn</sup>	30
C2	Clinical	-	+	+	-	0.632 ± 0.029 <sup>op</sup>	26
C13	Clinical	-	+	+	-	0.457 ± 0.058 <sup>uv</sup>	10
C17	Clinical	+	+	+	+	0.987 ± 0.128 <sup>f</sup>	57
C14	Clinical	-	+	+	+	0.289 ± 0.008 <sup>xy</sup>	-5
C15	Clinical	+	+	+	-	0.583 ± 0.016 <sup>qr</sup>	21
C16	Clinical		+	+	-	1.238 ± 0.088 <sup>e</sup>	80
C18	Clinical	-	+	+	-	1.318 ± 0.021 <sup>de</sup>	87
C19	Clinical	+	+	+	-	0.433 ± 0.004 <sup>w</sup>	8

Table 1. Contd.

Isolate	Source	Coagulase	Virulence genes present			Cytotoxicity	
			16S rRNA	<i>clfA</i>	<i>mecA</i>	LDH ( $A_{490/655}$ )	%
C28	Clinical	-	+	+	+	0.547 ± 0.017 <sup>op</sup>	18
C22	Clinical	+	+	+	-	0.154 ± 0.006 <sup>yz</sup>	-17
C24	Clinical	+	+	+	-	0.501 ± 0.069 <sup>t</sup>	14
C10-3	Clinical	-	+	+	-	1.614 ± 0.018 <sup>bc</sup>	113
C4	Clinical	+				0.531 ± 0.006 <sup>rs</sup>	16
C6	Clinical	NT	+	+	+	0.776 ± 0.036 <sup>ij</sup>	38
C7	Clinical	+	+	+	+	0.663 ± 0.008 <sup>no</sup>	28
C9	Clinical	-	+	+	-	0.378 ± 0.011 <sup>y</sup>	3
C29	Clinical	-	+	+	+	NT	
C5	Clinical	-	+	+	+	NT	
C12	Clinical	NT	+	+	+	NT	
C27	Clinical	NT	NT			NT	
C26	Clinical	NT	NT			NT	
ATCC 8325-4		NT	+	+	+	0.840 ± 0.050	44

a = Virulence genes detected by PCR; b=The LDH release values are averages ± standard deviations for three replicates. Means with same alphabets are not different ( $p < 0.05$ ); c NT=Not tested



**Figure 2.** PFGE patterns of *Sma*I restriction enzyme digested DNA of *S. aureus*. Lanes 1 to 5 are representative of PFGE pattern from clinical (A) and milk (B) strains; lanes 1, 7 and 15 correspond to ATCC 700698; lanes 2 to 6 and 8 to 13 correspond to milk strains.

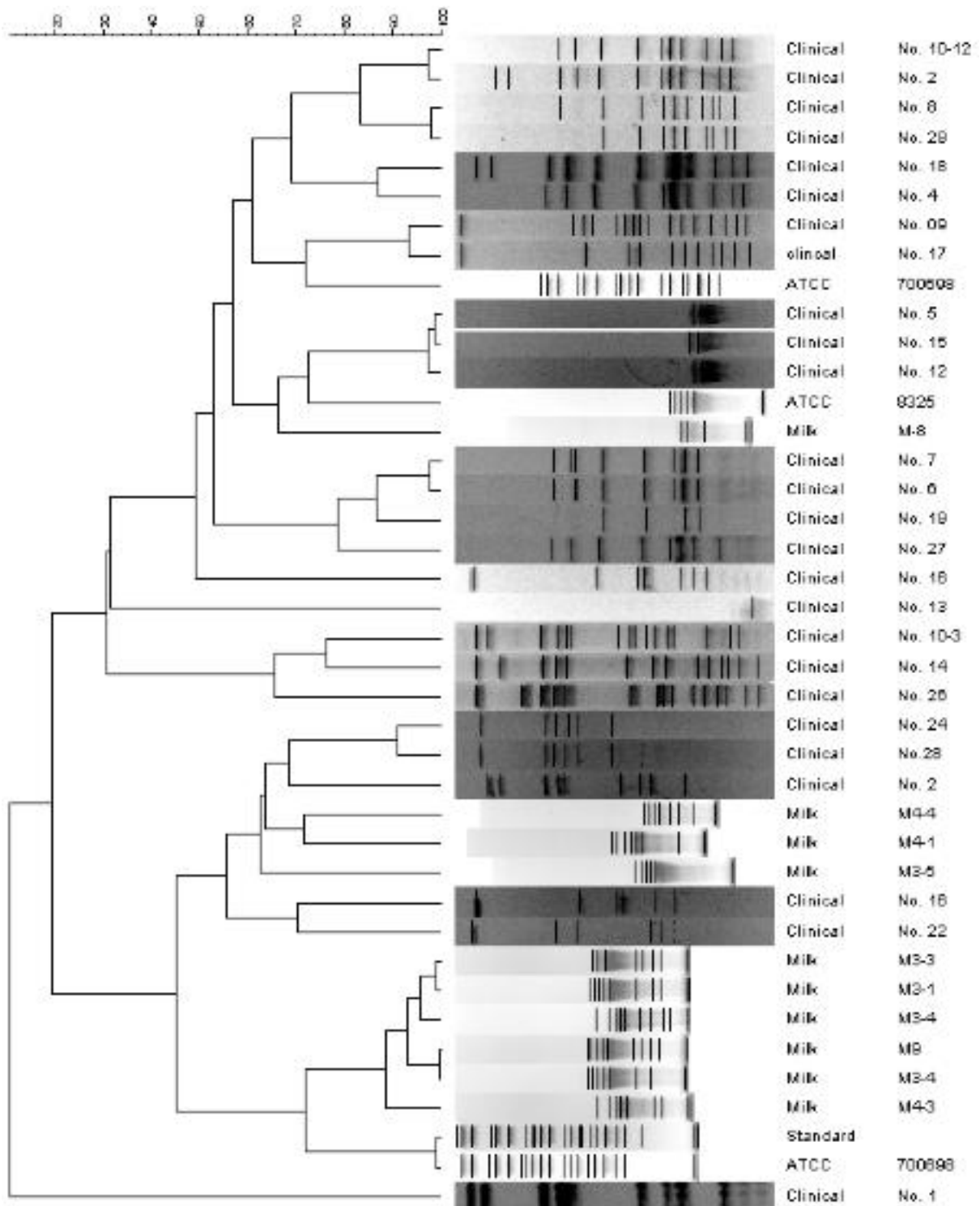
isolates, and also between meat and milk isolates as shown in Figure 5. However, there was no significant difference ( $p < 0.05$ ) in LDH release between the clinical and meat isolates. The meat isolates displayed the widest range of LDH release, followed by the clinical isolates. However, the clinical isolates had the smallest number of isolates with negative LDH release. This assay is used to assess the pathogenic capabilities of the strains isolated from milk, meat and clinical sources.

## DISCUSSION

In this study, 61 *S. aureus* strains isolated from milk, rabbit meat and human infections were investigated for

16S rRNA gene and *clfA* and *mecA* virulence genes by PCR. PCR techniques provide increased sensitivity, allow for more rapid laboratory processing times and enhance the likelihood of detecting bacterial pathogens (Riyaz-UI-Hassan et al., 2008). It has also been proposed for detection of foodborne pathogens to replace the time consuming classical techniques (Olsen, 2000).

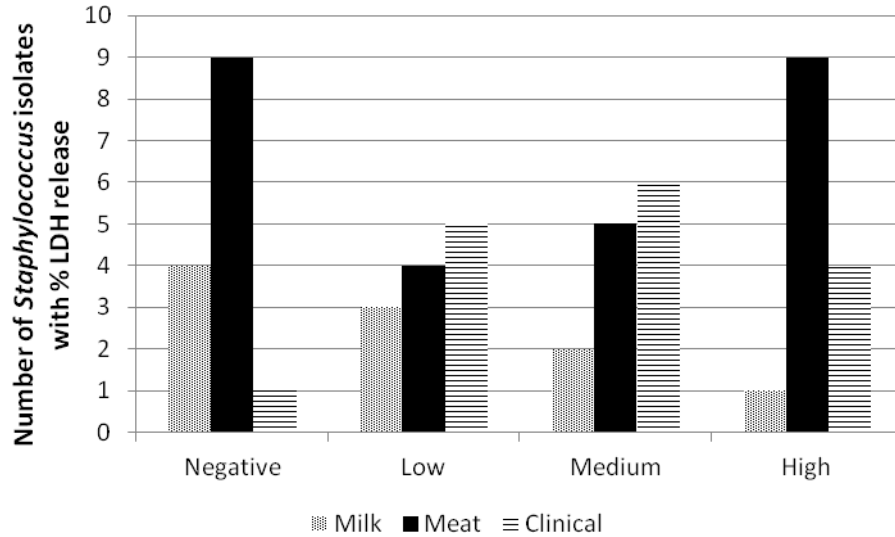
The strains were also genotyped using PFGE and the cytotoxicity profile was determined. All 61 isolates from this study were positive for 16S rRNA gene, as well as in previous studies (Mason et al., 2001; Zhang et al., 2004; Fan et al., 2008). The 16S rRNA gene is specific for *Staphylococcus* spp. Clumping factor (*clfA*) gene, was amplified for 20 of the clinical, 8 milk isolates and none of the strains isolated from rabbit meat. The isolation of



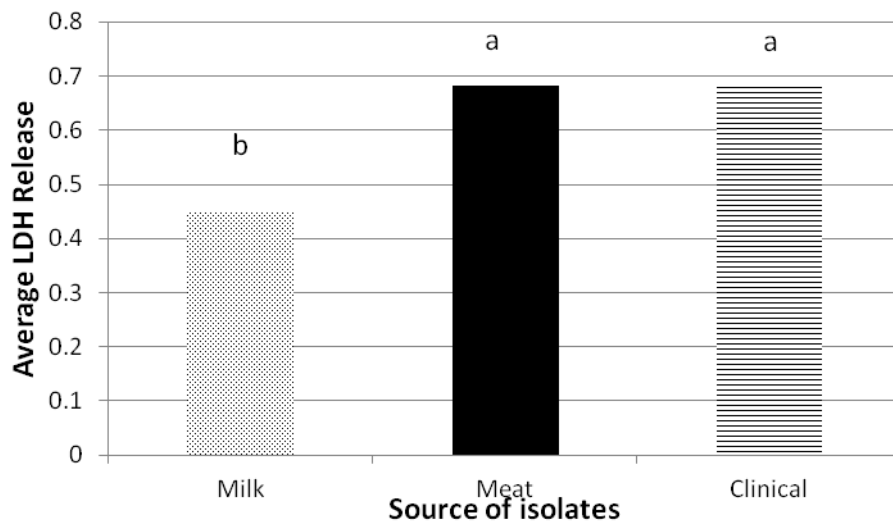
**Figure 3.** PFGE macro restriction profiles (SmaI) of *S. aureus* strains from clinical and milk sources. Pair comparison and cluster analysis performed with the Dice correlation coefficient and UPGMA.

clumping factor A is specific to *S. aureus*. Furthermore, *clfA* gene codes for a multifunctional protein, in addition to binding fibrinogen and it can also contribute to *S. aureus* capabilities of binding to platelet (Siboo et al.,

2001). *S. aureus* have also been documented to produce other fibrinogen binding proteins such as *clfB*, although the primary fibrinogen-binding protein is *clfA* (Ni Eidhin et al., 1998).



**Figure 4.** Percentages of cytotoxic *Staphylococcus* strains from milk, meat and clinical sources. Cytotoxicity values were grouped into negative (<1%); low (1 to 20%); medium (21 to 49%) and high (>50%) categories.



**Figure 5.** LDH release of *Staphylococcus* strains isolated from milk, meat and clinical sources. Mean separation is by Tukey's studentized analysis, means with same letters are not statistically different ( $p < 0.05$ ).

Pathogenesis of *S. aureus* results from the combined action of a diversity of factors, and infection begins with bacterial adhesion to host tissues (Jarraud et al., 2002). The *clfA* gene amplification results (87%) for the clinical strains in this study are comparable to previous studies by Tristan et al. (2003), who reported that *clfA* gene was present in 100% of the clinical samples tested while Peacock et al. (2002) reported 98% of *clfA* gene. Raw unpasteurized milk has been shown to be natural reservoir for *S. aureus* and other pathogenic microorganisms. In addition, studies have further demonstrated that

*S. aureus* can be isolated from raw milk, 43-62% of the times (Foschino et al., 2002; Chye et al., 2004). From the raw and unpasteurized milk procured for this study, 73% of the *S. aureus* isolates were positive for *clfA* gene. The emergence and increasing number of methicillin resistant *S. aureus* in foods and the environment are alarming. Furthermore, methicillin resistance gene in *S. aureus* is conserved and its amplification can be beneficial during outbreak investigation. Only 45% of clinical and 64% of milk strains was positive for *mecA* gene. Previously, it was reported by Suzuki et al. (1993) that in some strains

of *Staphylococcus* the loss of their regulator gene by deletion occurs and we speculate that in this research some clinical strains may have lost the regulator gene also. However, more of the strains isolated from milk had *mecA* gene. This poses a considerable public health risk, in particular because of the increase in number of individuals who consume raw milk (Riyaz-UI-Hassan et al., 2008). It is known that the food handlers are most often exposed to *S. aureus* contamination (Jablonski and Bohach, 2011; Jørgensen et al., 2005) and that raw milk and raw milk products from dairy animals, un-clean tanker and food handlers are probably equally involved as source of the contamination.

All the *S. aureus* strains from rabbit from this study were negative for the clumping factor A and methicillin resistance genes. According to Vancraeynest et al. (2006), a high virulence (HV) rabbit *S. aureus* clone causes transmission of chronic staphylococcosis, whereas low virulence (LV) strains only infect a limited number of animals. It is therefore speculated that the *Staphylococcus* strains from the rabbit isolates could be low virulent strains and that probably explains the inability of PCR to detect either clumping factor A or methicillin resistant gene.

All the strains were positive for 16S rRNA gene in *Staphylococcus* isolates. The PFGE was used to further discriminate the isolates. As reported by Revazishvili et al., (2006), PFGE is more useful for short-term studying of *S. aureus*; and is considered the gold standard technique for MRSA typing because of its high discriminatory power, its reproducibility, and its good correlation with epidemiologically linked data (Tenover et al., 1994). The results of the PFGE typing showed some degree of genetic heterogeneity among the *S. aureus* strains from clinical and milk sources. At 80% similarity level, 8 clusters were identified (Figure 3). The isolates from similar clinical or milk sources were grouped into the same PFGE cluster in this study in contrast to Xie et al. (2011) who reported same PFGE cluster organization from strains of *Staphylococcus* isolated from different sources. Five MRSA strains from clinical sources were not grouped into PFGE cluster while six strains isolated from milk were in one cluster.

Lactate dehydrogenase is a low molecular weight enzyme carried in the cytoplasm of the majority of cells, which is then released from the cells after a minor disturbance of the integrity of the cell membrane. The LDH results were comparable with LDH release in *E. coli*, using Vero cell assay (Maldonado et al., 2005), although in this study jejunal INT-407 cell culture was used to access the cytotoxicity of the *Staphylococcus* strains. The LDH release cytotoxicity assay from human intestinal cell line was applicable to determining the virulence capabilities of *Staphylococcus* strains isolated from food and clinical sources. Lactate dehydrogenase release also signified the pathogenic effect of *Staphylococcus* by causing cell damage and cell death of the INT-407.

However, the results of this study contradict Buxser and Bonventre (1981) study, which found that *Staphylococcus* enterotoxins A and B did not initiate LDH release in INT-407 cells.

Lactate dehydrogenase assay was performed to show the virulence potential of *Staphylococcus* isolates from milk, meat and clinical sources. According to Bhunia (2008) although *S. aureus* is generally not considered to be an intracellular pathogen associated with classical facultative intracellular pathogens such as *Listeria*, *Salmonella*, and *Shigella* spp., it has been documented that some *S. aureus* strains isolated from bovine mastitis can adhere and invade bovine mammary epithelial cell line MAC-T and primary bovine mammary secretory epithelial cells (Almeida et al., 1996; Bayles et al., 1998).

## Conclusion

These findings show the prevalence of *S. aureus* in food and clinical specimen, and we provide evidence that even if virulent genes such as *clfA* or *mecA* genes were not amplified in some strains, there may still be cytotoxicity effects presenting a public health concern particularly in individuals that consume game animals or raw milk and might have compromised immunity.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Full Length Research Paper

# Biosynthesis of thermostable $\alpha$ -amylase by immobilized *Bacillus subtilis* in batch and repeat batch cultures using fortified date syrup medium

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Batch and repeat batch fermentation were performed to evaluate the potential of date syrup and immobilized cells technique for the economical production of  $\alpha$ -amylase. The enzyme production by immobilized cells was greater than that of freely suspended cells. Khas date syrup gave the highest enzyme yield ( $142.7 \text{ Uml}^{-1}$ ) using the immobilized technique after 48 h of fermentation. The highest  $\alpha$ -amylase production was obtained in a culture grown in a 2% tricalcium phosphate treated medium. The enzyme production was proportional to the amount of date syrup up to 1.0% sugar. Tween 80 was more effective in enhancing cell membrane permeability, and thus increasing enzyme secretion. A 1.4 fold increase in enzyme production was achieved using an optimized medium in a fermenter. The culture activity under repeated batch cultivation remained stable up to the third cycle, and retained about 85% of its initial efficiency during the first five batches when each cycle continued for 48 h. The stimulated activity and the thermo stability were observed in the presence of  $10 \text{ mM Ca}^{2+}$ . The results show that date syrup is a promising economic carbohydrate source and cell immobilization is an excellent alternative method for enhanced  $\alpha$ -amylase production.

**Key words:**  $\alpha$ -Amylase, production, immobilization, *Bacillus subtilis*, date syrup.

## INTRODUCTION

Amylases are one of the most important industrial enzymes and account for nearly 25% of the world's enzyme sales (Rao et al., 2012). These enzymes are of great significance, with extensive biotechnological applications including starch degradation, detergent, food, pharmaceutical, textile and paper manufacturing (Acourene and Ammouche, 2010). Each application of  $\alpha$ -amylase (EC 3.2.1.1), requires unique properties with respect to specificity and thermal stability (Konsula and Liakopoulou-

Kyriakides, 2004). Improvement in the yield of  $\alpha$ -amylase and consequent cost reductions depends on the efficient utilization of agro-industrial residues and by-products as production media (Saad, 2006; Shamala et al., 2012). Kingdom of Saudi Arabia (KSA) is a major date producing country and is ranked the second in the world as per FAO statistics 2010 (El-Habba and Al-Mulhim, 2013). Date syrup is a byproduct of the date industry. It is rich in carbohydrates (75%) with small amounts of protein (1.1%),

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fat (2.9%), macro-elements in the form of Ca, P, Na, K and Mg and microelements in the form of Fe, Zn, Cu, and Mn (Al-Farsi et al., 2007). The analysis of the sugar content of date syrup shows the presence of fructose (41%), glucose (39%) and traces of sucrose (Khiyami et al., 2008). It can be used as a sweetener and flavouring for human consumption and for microbial fermentation (ethanol, vinegar, antibiotics and single cell protein) because it is readily available and relatively low priced (Radwan et al., 2010).

The enzyme production is greatly affected by the presence of heavy metals which are contained in large amounts in date syrup, so various chemical techniques have been used to precipitate or chelate the ions which cause critical problems during fermentation (Roukas and Kotzekidou, 1997; Saad, 2006). The  $\alpha$ -amylase is produced by a wide variety of microorganisms, but on account of its industrial applications, the best commercial producer are *Bacillus licheniformis*, *Bacillus stearothermophilus* and *Bacillus amyloliquefaciens* (Kiran et al., 2012). There is considerable interest in improving the productivity and product economy of  $\alpha$ -amylase by using the immobilized cells of *Bacillus* strains (Singh and Verma, 2008). Cells have been commonly entrapped in a calcium alginate gel through which substrates and products can diffuse easily (Gökhan et al., 2005). The use of immobilized cells offers several advantages over free cells, such as the relative ease of product separation, the re-use of biocatalysts, the prevention of washout, the reduced risk of contamination and operational stability (Shivakumar, 2012). Furthermore, using the entrapment technique, a dense cell culture can be established, leading to improved productivity (Poddar et al., 2011). In the present study, the potential of date syrup and immobilized cells technique for the  $\alpha$ -amylase production by batch and repeat batch fermentation were investigated.

## MATERIALS AND METHODS

### Source of strain

*Bacillus subtilis* EM11, identified by phylogenetic analysis, was isolated from soil samples collected from the Jazan region, Saudi Arabia, as described in our previous report (Alamri, 2010).

### Pre-treatment of crude date syrup

Date syrup was obtained from two companies in Saudi Arabia, Al-Barak (khlis date syrup, 70% sugars) and Durrah Dates (mixed date syrup, 66% sugars). Date syrup solution was diluted with distilled water in order to obtain 1% (w/v) sugar concentration, and treated with the following methods: 1- Tricalcium phosphate treatment (TCP): date syrup solution was adjusted to pH 7.0 with 1 N NaOH and treated with 1, 2 or 3% (w/v) TCP. The mixture was heated at 100°C for 5 min. The mixture was cooled and then centrifuged at 4,000 g for 20 min. 2- Potassium ferrocyanide (PFC): date syrup solution was adjusted to pH 7.0 with 1 N NaOH and sterilized at 121°C for 15 min. The liquid was treated while hot with 25 or 50  $\mu$ g/ml potassium ferrocyanide to encourage the precipitation of

heavy metals. 3- Methanol concentration: different methanol concentrations (1, 2, 3 and 4% v/v) were added to the fermentation flasks.

### Inoculum preparation

Five millilitres of sterile distilled water was added to a 24 h old slant of *B. subtilis*. The resulting cell suspension was transferred into 250 ml Erlenmeyer flasks containing 50 ml of Luria Bertani medium: 1% tryptone, 0.5% yeast extract and 0.5% NaCl, and cultivated at 45°C /200 rpm on an incubation shaker for 24 h. Cells were harvested by centrifugation (4500 rpm, 20 min), and were used both for immobilization and in experiments with free cells (Konsoula and Liakopoulou-Kyriakides, 2004).

### Immobilization

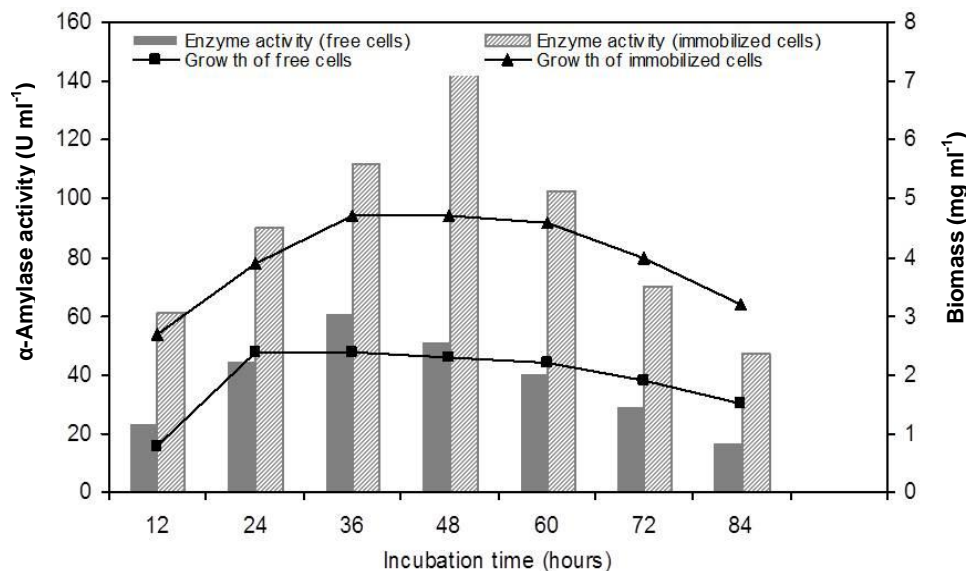
The alginate entrapment of cells was performed according to the method suggested by Konsoula and Liakopoulou-Kyriakides (2006). About 20 mg of wet cells and 12.5 ml of 2% (w/v) sodium alginate solution were mixed and stirred for 10 min to obtain a uniform mixture. The mixture obtained was extruded drop-wise through a 10 ml syringe into a 25 ml of 3.5% (w/v)  $\text{CaCl}_2$  solution. Alginate drops were solidified upon contact with  $\text{CaCl}_2$  forming capsules, and thus entrapping the bacterial cells. The capsules were allowed to harden for 30 min and then were washed with sterile saline solution (0.9% NaCl) 3 to 4 times to remove excess  $\text{Ca}^{2+}$  and cells. When the capsules were not used, they were preserved in a  $\text{CaCl}_2$  solution in the refrigerator. All operations were carried out aseptically within a laminar flow unit

### Fermentation

Fermentation was done in 250 ml Erlenmeyer flasks, each containing 50 ml of date syrup medium (1% sugar) fortified with peptone 0.5%,  $\text{K}_2\text{HPO}_4$  0.6%,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.02% and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.05% (Aqeel and Umar, 2010). Each flask was inoculated with the capsules obtained from 12.5 ml of alginate gel. For the free cell cultures, the production medium was inoculated with bacterial cells equivalent to those used in the immobilized cultures. The fermentation with free and immobilized cells was conducted at 45°C for 84 h under shaking conditions (200 rpm). For evaluation of the effect of surfactants on amylase production, 0.5% of different filter sterilized surfactants (sodium dodecyl sulphate (SDS), Triton-X100, Tween 40 and Tween 80) were added separately to the production medium. A 7.5 L jar fermenter (BioFlo/CelliGen 115 Benchtop Fermentor & Bioreactor) was employed for the fermentor operation with a culture volume of 2.5 L. The agitation speed and incubation temperature were controlled at 200 rpm, 45°C. The culture pH in the fermenter was controlled at 7.5 with an air flow of 4 v/v/min.

### Repeated batch fermentation with free and immobilized cells

One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of cells immobilized in an alginate matrix was examined. Repeated batch fermentations were conducted by running the fermentation for 48 h. At the end of each cycle, the production medium was recovered, and the immobilized cells were washed with 2% (w/v)  $\text{CaCl}_2$  solution for 30 min, fresh production medium was added and the fermentation was continued. Curing the gel capsules with 2%  $\text{CaCl}_2$ , after each fermentation batch prevented the disruption of the capsules, maintained their mechanical structure and significantly increased the productive life of the



**Figure 1.** Enzyme production by free and immobilized cells using khlas date syrup.

biocatalysts (Konsoula and Liakopoulou-Kyriakides, 2006).

All analyses were performed at  $p \leq 0.05$  using MINITAB, version 13.1.

### Biomass

Both cell growth in freely suspended cultures and immobilized cells in alginate matrix were determined as dry weight. The alginate capsules were dried and the biomass was determined from the difference between the total weight of the alginate gel capsules containing cells and that of alginate capsules without cells (blank) prepared under the same conditions of immobilization.

### $\alpha$ -Amylase assay

$\alpha$ -Amylase was assayed by adding 1 ml of enzyme to 1 ml soluble starch (1%) in an acetate buffer with pH 5, and incubated at 50°C for 15 min. The reaction was stopped by the addition of 2 ml of a 3,5-dinitrosalicylic acid reagent (Bernfeld, 1955). The absorbance was measured using a double beam UV/Vis scanning spectrophotometer (Model: Shimadzu, 1601PC) at 550 nm. One enzyme unit (U ml<sup>-1</sup>) is defined as the amount of enzyme which releases 1  $\mu$  mole of glucose.

### Effect of temperature on the activity and stability in the presence and absence of 10 mM CaCl<sub>2</sub>

The optimum temperature of the enzyme was evaluated by assaying activity between 40 and 100°C for 15 min in the presence and absence of 10 mM CaCl<sub>2</sub>. Thermal stability was investigated by measuring the residual activity after 60 min of pre-incubation at temperatures ranging from 40 to 100°C in the presence and absence of 10 mM CaCl<sub>2</sub> (Asgher et al., 2007).

### Statistical analysis

Firstly, parametric testing was performed and, then, the analysis of variance was used to compare the data from the different treatments.

## RESULTS AND DISCUSSION

### Enzyme production by free and immobilized cells using khlas date syrup

As shown in Figure 1, rapid cell growth in the free culture was observed in the first 24 h of cultivation, and a constant cell concentration (2.4 mg ml<sup>-1</sup>) was attained thereafter. On the other hand, the rapid increase in biomass entrapped in the gel capsules continued for 36 h, reaching a cell concentration of 4.7 mg ml<sup>-1</sup>. The time required for the stabilization of the cell content in the gel capsules was extended to 60 h of cultivation. The productivity of  $\alpha$ -amylase in terms of the immobilized cells was significantly greater than that of the freely suspended cells (about 2.35 fold). Although maximal enzyme production was attained after 36 h for the free cells, it was achieved after 48 h of cultivation for the immobilized cells. Similar observations have been reported for various *Bacillus* species. Most researchers attribute the alteration of the enzyme synthesis mechanism to the stress conditions imposed by immobilization, and to the changes in micro-environmental conditions, and to some metabolic and morphological alterations in the cells (Górecka and Jastrzębska 2011)

On the other hand, other researchers have suggested that immobilized biocatalysts produce lower levels of enzyme in comparison with free cells due to diffusion barriers and reduced oxygen availability to immobilized aerobic cells (Konsoula and Liakopoulou-Kyriakides, 2004).

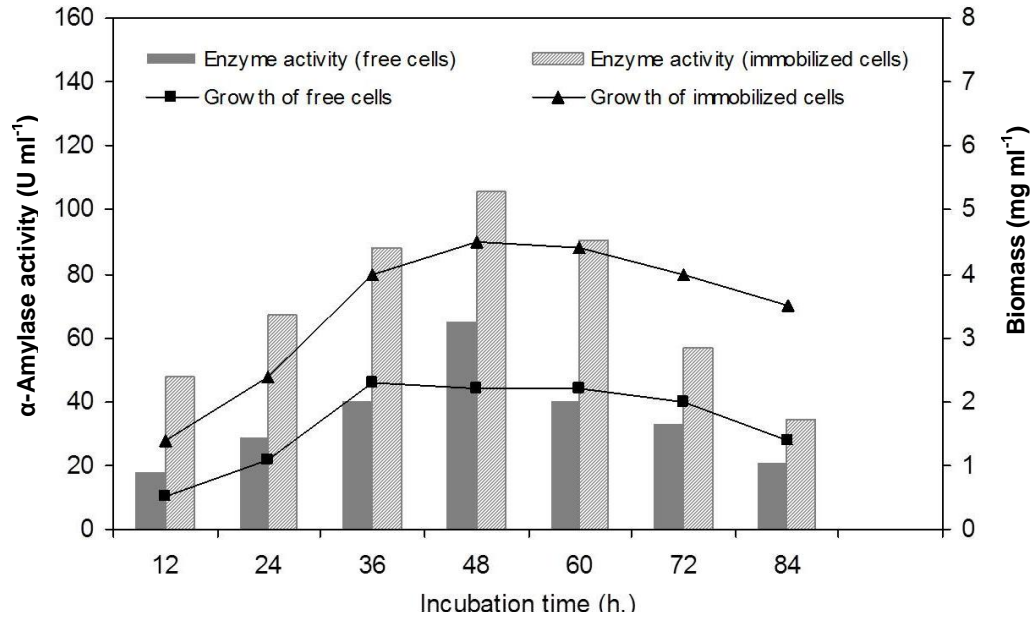


Figure 2. Enzyme production by free and immobilized cells using mix date syrup.

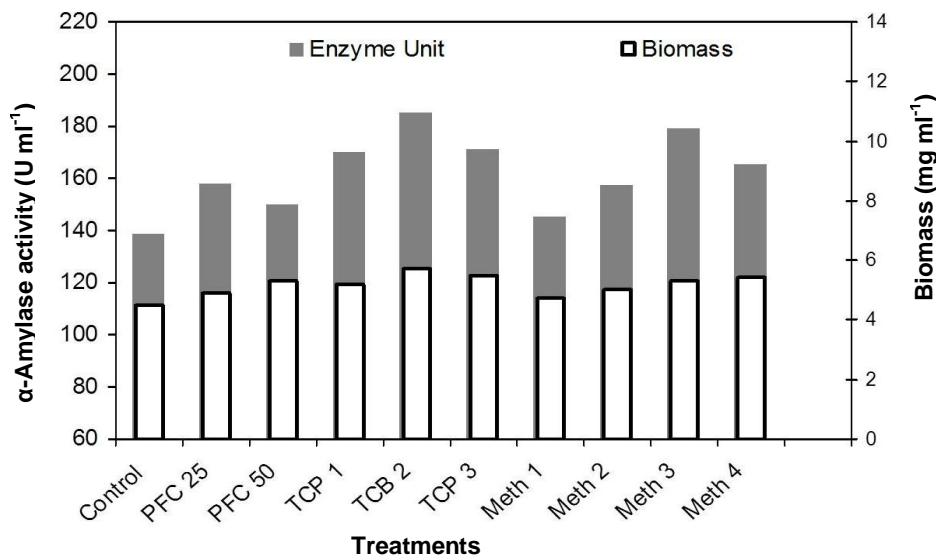
### Enzyme production by free and immobilized cells using mixed date syrup

The results illustrated in Figure 2 indicate that the growth of cells entrapped in a calcium alginate matrix increased gradually up to 48 h of incubation, whereas with free cells, gradual growth was observed only up to 36 h. Although maximal enzyme production was attained after 48 h of cultivation in both cases, a difference in the kinetics of  $\alpha$ -amylase biosynthesis was observed. The enzyme production was higher with immobilized cells ( $105.5 \text{ U ml}^{-1}$ ) than with free cells ( $65.3 \text{ U ml}^{-1}$ ). The cell immobilization technique results in a higher production rate within the first 24 h of cultivation (63.7%), while in the case of free cells, only 44.5% was produced during the same period. After 36 h of growth, the immobilized cells produced more than 83.8% of the maximal  $\alpha$ -amylase yield, while the production by free cells was restricted to 61.5%.

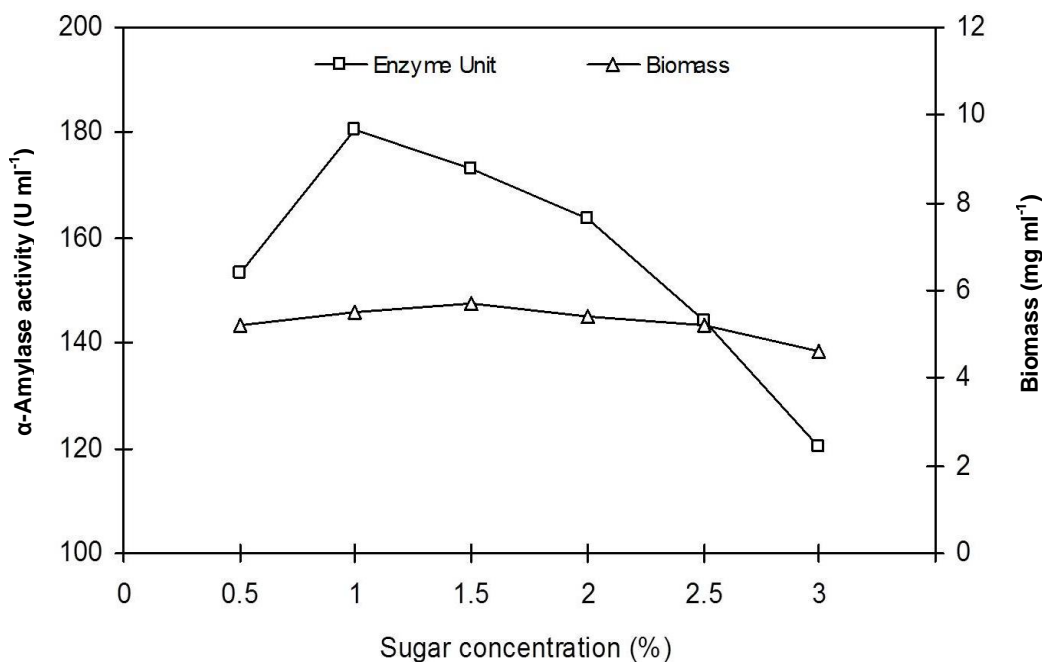
The increased  $\alpha$ -amylase production by the immobilized cells of *B. subtilis* may be due to the presence of a higher concentration of calcium ions as well as to the formation of strong gel capsules possessing high substrate mass transfer rates and low rates of cell leakage, as reported by Konsoula and Liakopoulou-Kyriakides (2006). Furthermore, according to Acourene et al. (2013),  $\alpha$ -amylase production is induced by the presence of starch in the production medium, which unfortunately cannot be used efficiently as a substrate due to the decreased mass transfer rate of this polysaccharide. In our study, the effect on the substrate mass transfer rate was reduced by the replacement of starch with date syrup.

### Effect of the pre-treatment of crude date syrup on enzyme production

Khlas date syrup was treated using different chemical methods to precipitate the heavy metals which affect  $\alpha$ -amylase production. The results presented in Figure 3 indicate that all treatments gave a remarkable increase in  $\alpha$ -amylase production and bacterial growth, in comparison with the results involving untreated date syrup. The highest value of  $\alpha$ -amylase production was obtained in a culture grown in 2% (w/v) tricalcium phosphate treated medium. The production of  $\alpha$ -amylase using a pre-treated date syrup medium was 33.8% higher than that associated with the untreated one. These results are possibly attributed to the presence of two phosphate groups rich in negatively charged oxygen atoms in tricalcium phosphate, that act as attractants for the positively charged elements present in molasses as reported by Mayilvahanan et al. (1996). They reported also that the pre-treatment with tricalcium phosphate dramatically reduced the concentrations of a number of heavy metal ions that may retard the utilization of molasses. 3% (v/v) methanol was the second best date syrup treatment. The high stimulation effect of methanol can be attributed to the increase in the microorganisms' tolerance to high levels of heavy metals (Mehyar et al., 2005). Furthermore, Mostafa and Alamri (2012) reported that undesirable substances such as iron, zinc and copper which are contained in great amounts in date syrup can cause critical problems during the fermentation process. It inhibits the growth of microorganisms, influences the ionic strength, the pH of the medium and is involved in the inactivation of the enzymes associated with metabolism.



**Figure 3.** Effect of pretreatment of crude date syrup on enzyme production. (Control: untreated, PFC 25 and 50: Potassium ferrocyanide (25 and 50 ug/ml), TCP 1, 2, 3: Tricalcium phosphate at 1, 2 and 3%, Meth 1, 2, 3, 4: Methanol at 1, 2, 3 and 4%).



**Figure 4.** Effect of sugar concentration of date syrup on enzyme production.

#### Effect of sugar concentration on enzyme production

The data presented in Figure 4 indicates that the date syrup was thought to be a good carbon source and is sufficient to support microbial growth and  $\alpha$ -amylase production. The maximum production of  $\alpha$ -amylase was achieved at 1.0% sugar, and the productivity was propor-

tional to the amount of date syrup. A further increase in the sugar concentration did not improve the enzyme production. Swain et al. (2006) and Aqeel and Umar (2010) reported that the  $\alpha$ -amylase production by *B. subtilis* was constitutive, since the biosynthesis of the enzyme took place, not only in the presence of starch, but also with other carbon sources such as maltose, glucose, fructose

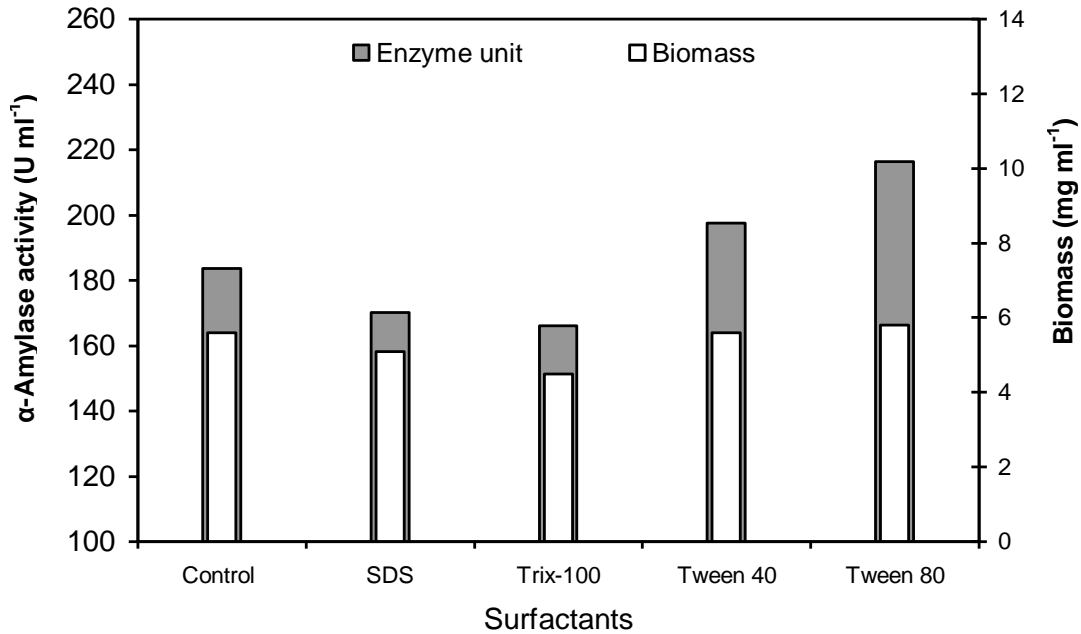


Figure 5. Effect of surfactants on enzyme production.

and date syrup. Pan and Xu (2003) and Radwan et al. (2010) found that the date syrup contains sufficient amounts of sugar that cover the growth of the strain. They reported also that the date syrup contains 39% glucose; therefore, glucose has no effect on the production of amylase in the presence of date syrup. Moreover, enzyme production reached the maximum yield after 48 h, which suggests that the catabolic repression of glucose did not occur. The results also indicate that the strain produced  $\alpha$ -amylase constitutively and without sensitivity to catabolite repression or transient repression. On the other hand, Sudharhsan et al. (2007) reported that the synthesis of carbohydrate degrading enzymes in some species of genus *Bacillus*, leads to catabolic repression by readily metabolizable substrates such as glucose and fructose.

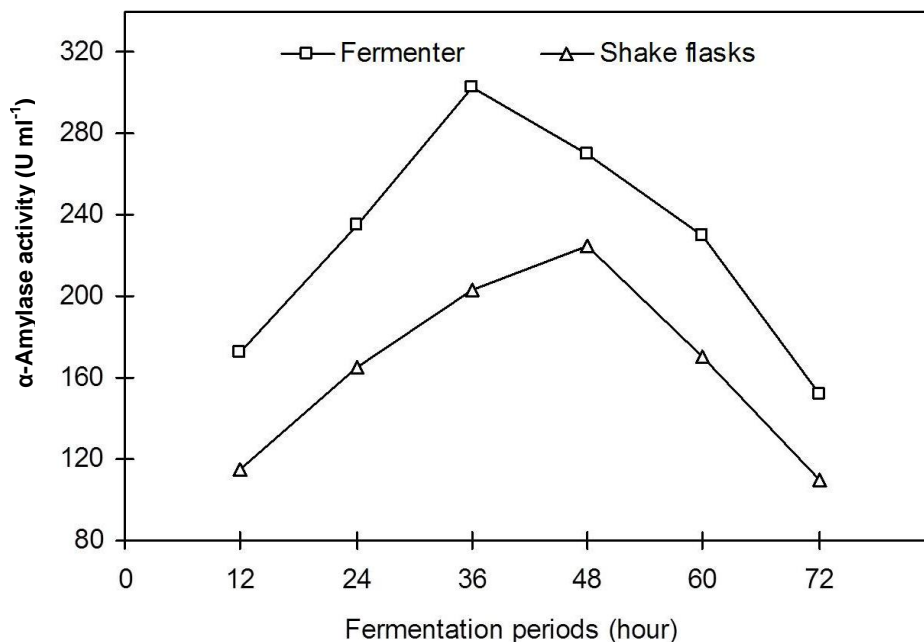
#### Effect of surfactants on enzyme production

The results illustrated in Figure 5 indicate that the addition of surfactants sometimes either increases or decreases enzyme production. The results shows clearly that the addition of a non-ionic detergent, Tween 80, causes the maximum production of  $\alpha$ -amylase (216.3 Uml<sup>-1</sup>) followed by Tween 40 (197.5 Uml<sup>-1</sup>) in comparison with the control. These results were in agreement with that of Sankaralingam et al. (2012). They reported that Tween 80 was an excellent surfactant for the production of  $\alpha$ -amylase. Surfactants reduce the surface tension of the liquid medium, and also provide essential nutrients for the growth of the organism, and increase the secretion of  $\alpha$ -amylase from the bacterial cells by increasing the cell

membrane's permeability (Uelger and Cirakoglu, 2001). The enzyme production was greatly inhibited by the addition of other surfactants, Triton X100 and SDS. It might have been due to the fact that sodium ions, along with sulphate ions, were toxic for bacterial growth and enzyme production as reported by Milner et al. (1996). Also, Serin et al. (2012) reported that Triton X-100 suppressed  $\alpha$ -amylase production in *Bacillus circulans*. On the other hand, Sudharhsan et al. (2007) found that the addition of SDS causes a higher production of amylase, but the other additives such as Triton X100 and Tween 20 decreased the production of  $\alpha$ -amylase.

#### Batch fermentation under optimized conditions in shake flasks and fermenter

To confirm the aforementioned performance of 2% tricalcium phosphate, 1% sugar, 0.5% Tween 80 with the basal medium components, fermentor operations were carried out using a 2.5 L working volume. Under optimized conditions, batch fermentation using shake flasks and a fermenter was carried out for up to 72 h (Figure 6). The production of  $\alpha$ -amylase in the fermenter after 36 h of cultivation was higher than that in the shake flask after 48 h, probably mainly due to the improvement in aeration conditions. In other words, there is great potential for an improvement in terms of  $\alpha$ -amylase yield by further optimizing the operating conditions in future experiments. An overall 1.34-fold increase in enzyme production was achieved using an optimized medium in a fermenter when compared with a shake flask. This might be due to the fact that this medium provided an adequate amount



**Figure 6.** Batch fermentation under optimized conditions in shake flasks and fermenter.

of essential nutrients for microbial growth, and subsequently for enzyme production. Crueger and Crueger (2000) reported that molasses is one of the cheapest sources of carbohydrates. Besides a large amount of sugar, molasses contain nitrogenous substances, vitamins and trace elements. Our results indicated that the evaluation of a suitable medium is critical for a successful fermentation process by microbes. Similar behaviour was reported by Uma et al. (2007). They found that a 1.6 and a 2.1-fold increase in enzyme production were achieved in an optimized medium in shake flasks and fermenter, respectively. Moreover, Bozic et al. (2011) reported that during the batch fermentation of *B. subtilis* IP 5832, a 60% higher  $\alpha$ -amylase activity was obtained.

#### Repeated batch fermentation with free and immobilized cells

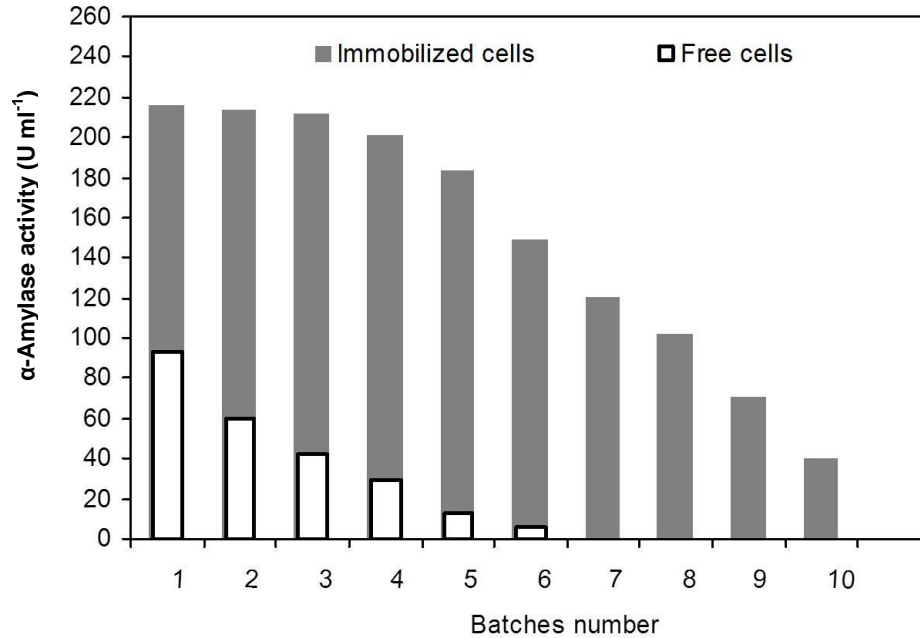
One of the most important benefits of immobilized cells is their ability in a stable fashion to produce  $\alpha$ -amylase under repeated batch cultivation. The  $\alpha$ -amylase production by *B. subtilis* cells for 10 batch cultivations with parallel experiments using free cells as a control are recorded in Figure 7. The results indicate that the free cells lost 35.4% of their productive ability after the first batch. In contrast, the immobilized cells showed high  $\alpha$ -amylase productivity upon re-use. The culture activity remains stable up to the third cycle, and retains about 85% of their initial efficiency during the first five batches when each cycle was continued for 48 h. The enzyme production with immobilized cells gradually decreases from the fifth batch onwards. This may occur as a result of beads disin-

tegrating during the batch operation. Thus, the repeated batch fermentation with calcium alginate beads was successfully run for 5 batches (10 days). The repeat batch culture technique has many advantages over batch cultivation, such as decreases in the cost of sterilization and the preparation of a fermenter and inoculums preparation. This increases the economic efficiency of enzyme production (Ates et al., 2002). The enzyme production after 5 fermentation cycles showed that the  $\alpha$ -amylase yield could be raised from 237.8 units in a free cell state to 1,025.8 units using an immobilized state. These findings were similar to those reported by Kiran et al. (2012) and Konsoula and Liakopoulou-Kyriakides (2006). The lengthy viability of the immobilized cells may be due to the different composition of proteins, nucleic acids and inorganic substances, in comparison with the free cells (Shivakumar, 2012).

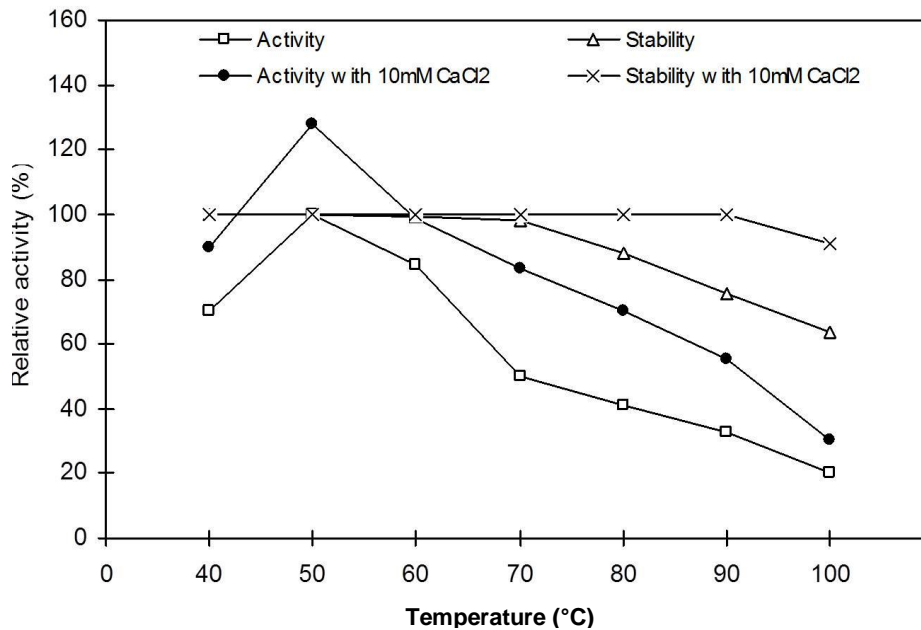
#### Effect of temperature on the activity and stability of $\alpha$ -amylase in the presence and absence of 10 mM $\text{CaCl}_2$

The  $\alpha$ -amylase activity was determined at different temperatures ranging from 40 to 100°C in the absence and presence of 10 mM  $\text{CaCl}_2$  (Figure 8). The optimum enzyme activity was recorded at 50°C, with the enzyme activity gradually declining at temperatures beyond this value. The enzyme activity sharply decreased to about 50.2% at 70°C. The activity of the enzyme was increased to 128% in the presence of 10 mM  $\text{Ca}^{2+}$ , suggesting that calcium is needed for the optimum activity of the enzyme. Most amylases are known to be metal ion-dependent





**Figure 7.** Repeated batch fermentation with free and immobilized cells.



**Figure 8.** Effect of temperature on the activity and stability of  $\alpha$ -amylase in the presence and absence of 10 mM  $\text{CaCl}_2$ .

enzymes. The calcium ion was reported to increase the amylase activity of *Bacillus* strains (Asgher et al., 2007). Furthermore, Gupta et al. (2003) and Rao et al. (2012) reported that the  $\alpha$ -amylase enzyme contains at least one  $\text{Ca}^{2+}$  ion and the affinity of  $\text{Ca}^{2+}$  is much stronger than that of other ions. The thermal stability of the  $\alpha$ -amylase was tested at different temperatures in a range of 40 to 100°C for a period of 1 h in the presence and absence of

10 mM  $\text{CaCl}_2$  (Figure 8). The enzyme was thermo-stable over a temperature range of 40 to 70°C, retaining 88.1% of its original activity at 80°C after 1 h of incubation in the absence of  $\text{CaCl}_2$ . The stimulated thermo-stability was observed in the presence of 10 mM  $\text{Ca}^{2+}$ . The enzyme was completely active up to 90°C, while 90.8% remained after pre-incubation at 100°C.

The stabilizing effect of  $\text{Ca}^{2+}$  on the thermo-stability of

the enzyme can be explained due to the salting out of hydrophobic residues by  $\text{Ca}^{2+}$  in the protein, thus causing the adoption of a compact structure (Swetha et al., 2006). Similarly, complete activity at 90°C for 1 h for the amylase from *Bacillus* sp. has been reported by Teodoro and Martins (2000). Violet and Meunier (1989) reported that  $\alpha$ -amylase contains at least one  $\text{Ca}^{2+}$  molecule which is involved in the stabilization of the molecular structure. Moreover, Konsula and Liakopoulou-Kyriakides (2004), Hwang et al. (2013) and Sindhu et al. (2011) found that the thermo-stability of  $\alpha$ -amylase from the *B. subtilis* and *Streptomyces avermitilis* strain was enhanced in the presence of calcium ion. The stability of the enzyme could be due to its genetic adaptability when it comes to carrying out their biological activities at higher temperatures. Our results confirm the key role of  $\text{Ca}^{2+}$  in terms of enzyme thermo-stability- an important feature for the use of amylolytic enzymes in starch-processing industries.

## Conclusion

The productivity of  $\alpha$ -amylase by the immobilized cells was greater than that of the freely suspended cells. The activity of the culture remained stable after a repeated batch culture for about five cycles. The potential application of  $\alpha$ -amylase in various industries and the need for the development of economic methods for improved production make date syrup an economic carbohydrate source, and whole bacterial cell immobilization, excellent alternative methods for enhanced amylase production.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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## Full Length Research Paper

## Characterization of epiphytic bacteria isolated from chickpea (*Cicer arietinum* L.) nodules

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Twenty eight isolates associated with the nodule surface of two chickpea cultivars, that is, reverted non-nodulating (ICC4993NN(R)) and normal chickpea (HC5) cultivars were isolated. All of them were found to be Gram positive. The isolates NE81, NE82 from cv ICC4993NN(R) and NE1043 from cv HC5 inhibited the growth of isolates NE84, NE44, NE45, NE32, NE16-1, NE32, NE84, NE44, NE19, NE26 and NE14, NE53, NE16-2, NE18, NE1043, respectively. However, some of these nodule epiphytic bacteria promoted the growth of mesorhizobial isolates from chickpea. The co-inoculation of these nodule epiphytes with mesorhizobial isolate resulted in significant increase in nodulation and shoot dry weight in chickpea as compared to the inoculation only with nodule epiphytes.

**Key words:** Chickpea, nodules, epiphyte, growth inhibition.

### INTRODUCTION

Rhizosphere is microbe and nutrient rich environment where the roots exude water and other compounds, thereby providing favourable environment for the growth of microorganisms. Rhizospheric microorganisms have different types of interactions with plant roots and nodules in the rhizosphere (Pinton et al., 2001; Werner, 2004; Hayat et al., 2010). These plant-microbe interactions influence the plant growth. Bacteria are predominantly found in the root rhizosphere and are commonly termed rhizobacteria (Antoun and Kloepper, 2001) but can also be referred to as root epiphytes. These root epiphytes can have beneficial, damaging or harmless effects on plant growth. In the last few decades, a large number of bacteria such as *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance plant growth (Kloepper et al., 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995). A total of 150 bacterial isolates belonging to *Bacillus*, *Pseudomonas*, *Azotobacter* and *Rhizobium* were reported from different

rhizospheric soil of chick pea in the vicinity of Allahabad, India (Joseph et al., 2007).

Significant increase in all the plant growth parameters were observed in maize and chickpea plants inoculated with *Pantoea agglomerans* (Mishra et al., 2011). Harmful rhizobacteria affect the growth of plants by producing metabolites like phytotoxins or phytohormones or by having competition for the nutrients (Nehl et al., 1996; Sturz and Christie, 2003; Kloepper, 2003). These rhizospheric bacteria also exhibit biocontrol activities. Two bacterial strains, *Pseudomonas* sp. and *Rhizobium* sp. obtained from the rhizosphere of chickpea have unique ability to inhibit *Fusarium oxysporum* f.sp. *ciceri*, *Rhizoctonia bataticola* and *Pythium* sp. under *in vitro* conditions (Nautiyal, 1997). Strong antagonistic activity was shown against three races of *F. oxysporum* f. sp. *ciceri* by 24 bacterial isolates and *Pseudomonas chlororaphis*. The extent of growth inhibition was influenced both by the bacterial isolates and the race of pathogen (Landa et al., 1997). A chitinase

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producing strain *Lysinibacillus fusiformis* was isolated from chickpea rhizosphere. It exhibited *in vitro* antifungal activity against a wide range of fungal plant pathogens and several plant growth promoting rhizobacteria (PGPR) activities yet exact mechanisms are not known (Singh et al., 2012). However, environmental conditions, soil conditions, growth of host plants or host genotype may also have some effect.

Leguminous plants have symbiotic association with the nitrogen fixing bacteria in their roots nodules. These bacteria are capable of converting atmospheric nitrogen into ammonia which is then incorporated into plants in the form of amino acids. ICRISAT, Hyderabad, India developed a non-nodulating variety of chickpea to estimate the N<sub>2</sub> fixation purely by plants (Rupela, 1994). These non-nodulating selections reverted back to nodulating selections only in certain locations particular at Hisar, Haryana, India (Dudeja et al., 1997). Now pure lines of reverted lines of chickpea from non-nodulating to nodulating ones are available at Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India. The nodules of this revertant non-nodulating chickpea cultivar (ICC4993NN(R)) as well as normal chickpea cultivar (HC5) were used to study the epiphytic bacteria associated with them, to have an insight into their effect on host plant. The present investigation was undertaken with the objective of isolating and comparing the different characteristics of epiphytic bacteria associated with the nodules of reverted non-nodulating (ICC4993NN(R)) and normal chickpea (HC5) cultivars.

## MATERIALS AND METHODS

### Isolation of epiphytic bacteria associated with nodules of reverted and normal chickpea cultivars

Reverted non nodulating lines of cv ICC4993NN(R) showing nodulation and normal nodulating cv HC5 were grown under field conditions at research farm of Chaudhary Charan Singh Haryana Agricultural University, Hisar, India, and were then uprooted on the 60<sup>th</sup> day of growth. The healthy nodules formed by non-nodulating line and other normally nodulated cultivars were removed and used for isolation. The nodules were mildly surface sterilized with 0.1% HgCl<sub>2</sub> and kept on Tryptone Soya Agar (TSA) plates as sterilization with 0.2% HgCl<sub>2</sub> resulted into complete sterilization of nodules (Vincent, 1970). Growth around these partially sterilized nodules was observed and isolates were picked up from the plates.

### Study of morphological characters of nodule epiphytes of reverted and normal chickpea cultivars

The different morphological characters such as growth, colony colour, appearance, morphology, gum production of all the isolated nodule epiphytes were observed. Gram staining of nodule epiphytes was done and cell shape whether cocci or rods were observed under the microscope. These were further used to study their beneficial and inhibitory effects on the growth of mesorhizobial isolates and on each other, respectively. The inhibitory effect was observed by zone of inhibition test. A TSA plate was inoculated with a lawn of a bacterial culture suspension. The test organism was

placed in the well. After incubation for 24 h, the plate was examined to check the inhibition of growth of test organism. The beneficial effect was observed by paired test. The test organism and the isolated mesorhizobia from the nodules of chickpea were streaked on the same plate. Following incubation, these plates were observed after 24 h. Those nodule epiphytes which were showing positive effect on the growth of mesorhizobia were further selected for pot experiment.

### Effectiveness of nodule epiphytes

The pot experiment was conducted using 10 nodule epiphytes selected on the basis of their growth promotion activity in paired test. Nodule epiphytic isolates (NE32, NE292-1, NE45, NE81, NE12, NE13, NE82, NE16-1, NE53 and NE42) were inoculated separately in TSA broth and the mesorhizobial isolate (NN95) was inoculated in yeast extract mannitol (YEM) broth and incubated on rotary shaker at 28±2°C in an incubator. River sand was thoroughly washed with acid followed by 6-7 washings with water and was sterilized in oven at 180°C for one hour in trays. The sand was put in cups and nitrogen free nutrient solution was added and cups were covered with paper, held in position with the help of a thread. These test assemblies were sterilized in autoclave at 15 lb/in for one hour. Few empty cups were also sterilized in autoclave at 15 lb/in for 20 min and were used whenever needed to support the cups containing sand. In one set of experiment, germinated seedlings of chickpea were transferred to sterilized cups containing sand, together with 1-2 mL broth of nodule epiphytes only and control was kept without any inoculum. In another set of experiment, the germinated seedlings were placed in the test assemblies and co-inoculated with 1-2 mL broth of nodule epiphytes and 1-2 mL broth of chickpea mesorhizobial isolate and control was inoculated only with *Mesorhizobium*. Then, these nodulation test assemblies were kept in green house and watered daily with sterilized Sloger's nitrogen free watering solution. After 55 days of growth, plants were recovered and analyzed for nodulation and other parameters. For determining dry weight of shoot and root, samples were dried in an oven at 80°C for 48 h or till the constant weight was observed (Somasegaran and Hoben, 1994).

## RESULTS

The surface sterilization methods were evaluated for standardization of isolation process of epiphytes. The nodules when, mildly surface sterilized with 0.1% HgCl<sub>2</sub> and placed on TSA plates showed growth around these partially sterilized nodules (Figure1a). However, with 0.2% HgCl<sub>2</sub> (Figure1b) there was complete sterilization of nodules, as no epiphytic growth was observed.

In total, 28 Gram positive nodule epiphytes were obtained from both cultivars (Table 1). They showed large variation in colony colour ranging from white, cream, pale white, light brown, yellow to red. The cell shape also varied from cocci, small rods to long rods. Some isolates produced gum while others did not. All the isolates were assessed for their growth inhibition activity against other isolates. The isolates NE81, NE82 (ICC4993NN(R)) and NE1043 (HC5) showed zone of inhibition against other isolates. The isolate NE81 showed zone of inhibition against isolate NE84, NE44, NE45, NE32 and NE16-1 (Figure 2a and b) and NE82 showed inhibition against NE32, NE84, NE44, NE19 and NE26 (Figure 3a and b).



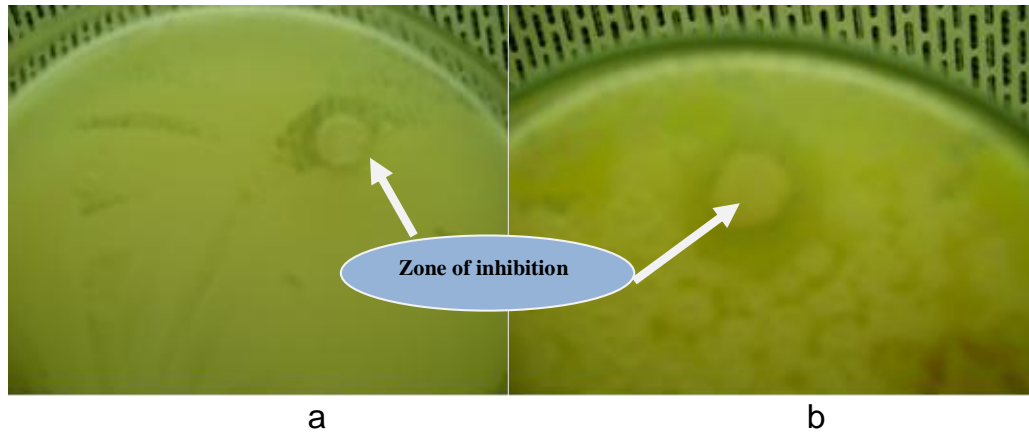
**Figure 1.** Presence (a) and absence (b) of rhizospheric growth around nodules after sterilization with 0.1 and 0.2% HgCl<sub>2</sub>.

**Table 1.** Morphological characters of rhizospheric bacterial epiphytes from chickpea nodules.

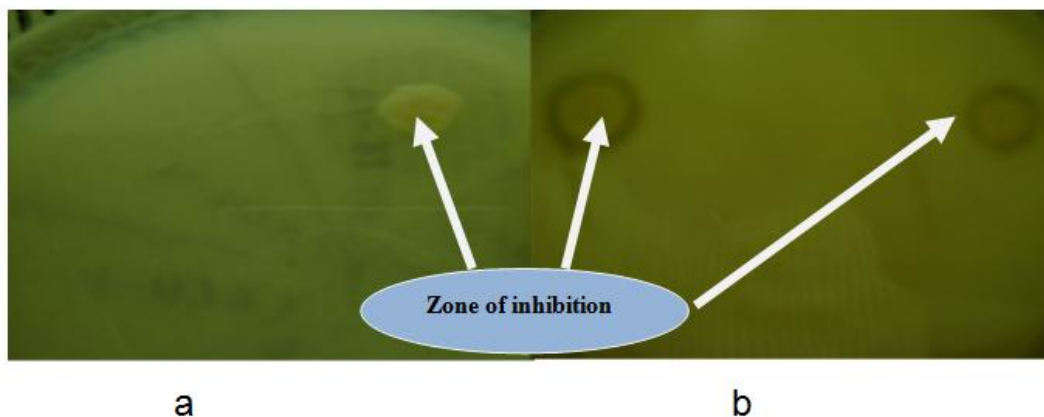
<b>Nodule epiphyte</b>	<b>Colony morphology</b>	<b>Gram staining</b>
NE12	Cream, gummy, large, round, long rods	Gram +ve
NE13	Light brown, gummy, large, round, long rods	Gram +ve
NE14	Cream, non gummy, irregular and flat, small rods	Gram +ve
NE15	Light brown, gummy, irregular, small rods	Gram +ve
NE16-1	Light brown, gummy, irregular and raised, small rods	Gram +ve
NE16-2	Cream, gummy, medium, round, long rods	Gram +ve
NE18	Pale white, non gummy, uneven and raised, small rods	Gram +ve
NE19	Cream, gummy, irregular, long rods	Gram +ve
NE26	Light brown, gummy, uneven, medium rods	Gram +ve
NE32	Cream, gummy, round and raised, small rods	Gram +ve
NE42	Red, gummy, medium, round, cocci	Gram +ve
NE44	Yellow, gummy, small, round, cocci	Gram +ve
NE45	Light brown, gummy, small, round, cocci	Gram +ve
NE53	Cream, gummy, round, medium rods	Gram +ve
NE67	Yellow, non gummy, round, cocci	Gram +ve
NE81	Cream, non gummy, large, uneven and flat, long rods	Gram +ve
NE82	White, non gummy, small, rhizoid and raised, medium rods	Gram +ve
NE83	White, gummy, medium, round, small rods	Gram +ve
NE84	Brown, gummy, irregular, medium rods	Gram +ve
NE281	Brownish cream, gummy, irregular, rods	Gram +ve
NE292-1	White, non gummy, irregular, small rods	Gram +ve
NE296	Yellow, gummy, small, medium rods	Gram +ve
NE297	Pink, slight, gummy, round, medium rods	Gram +ve
NE1006	Cream, non gummy, irregular, small rods	Gram +ve
NE1043	Cream, non gummy, round, small rods	Gram +ve
NE1042	Pale white, non gummy, round and raised, small rods	Gram +ve
NE1044	Cream, gummy, round, small rods	Gram +ve
NE1016	Light yellow, gummy, irregular, medium rods	Gram +ve

The growth of NE14, NE53, NE16-2, NE18 and NE1006 was inhibited by isolate NE1043 (Figure 4a and b) (Table

2). Interestingly, one of the isolate (NE81) was found to solubilise the media components and resulted in clearing



**Figure 2.** Inhibition activity shown by the nodule epiphyte NE 81 against other nodule epiphytes, (a) NE32 and (b) NE45.

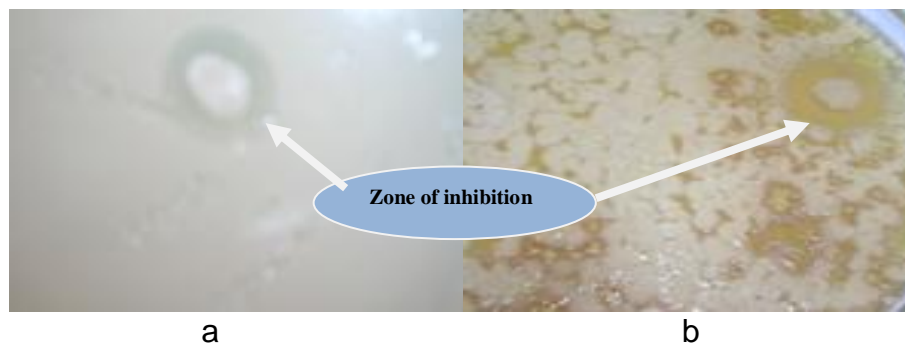


**Figure 3.** Inhibition activity shown by the nodule epiphyte NE 82 against other nodule epiphytes, (a) NE 44 and (b) NE32.

zone (Figure 5).

Out of 28, 10 nodule epiphytes showed the growth promotion activity towards mesorhizobial isolate NN95. The growth promotion activity of two isolates NE 82 and NE 81 is depicted in Figure 6a and b, respectively. The germinated chickpea seeds were inoculated with nodule epiphytes alone as well as co-inoculated with mesorhizobia and nodule epiphytes. After 55 days of growth, plants were uprooted for nodulation, root and shoot biomass (Figures 7 to 9). Nodule epiphytes when inoculated alone showed no nodulation or nodulation less than five nodules per plant except the isolate NE16-1. When chickpea seeds were co-inoculated with *Mesorhizobium* and nodule epiphytes, there was significant increase in nodulation. The results showed that there was no nodulation in control but when inoculated with only nodule epiphytes nodulation ranged from 0 to 7 nodules per plant. Mesorhizobial isolate alone resulted in 15 nodules per plant. The nodulation ranged from 14 to 34 nodules per

plant when plants were co-inoculated with *Mesorhizobium* and nodule epiphytes. Highest nodulation was observed in the case of plants co-inoculated with NE292-1 and *Mesorhizobium* (34 nodules per plant) followed by NE81 and *Mesorhizobium* (31 nodules per plant) and NE 32 and *Mesorhizobium* (27 nodules per plant). Shoot dry weight of control plants was 189 mg/plant, whereas it varied from 407 to 965 mg/plant in the case of nodule epiphytes from cv. ICC4993NN(R) and HC5. Highest shoot dry weight was observed in plants inoculated with NE12 followed by NE13, NE81 and NE32. Shoot dry weight varied from 623 to 1320 mg/plant in the case of plants co-inoculated with *Mesorhizobium* and nodule epiphytes. The increase in shoot dry weight was highly significant when seeds were co-inoculated with isolates NE12, NE16-1, NE82 and *Mesorhizobium* ( $P < 0.01$ ). Four isolates, NE292-1, NE42, NE53 and NE81 showed significant increase in shoot dry weight when they were co-inoculated with *Mesorhizobium* ( $P < 0.05$ ).



**Figure 4.** Inhibition activity shown by the nodule epiphyte NE 1043 against other nodule epiphytes, (a) NE14 and (b) NE18.

**Table 2.** Inhibitory effect of NE81, NE82 and NE1043 against thirteen rhizospheric bacterial epiphytes.

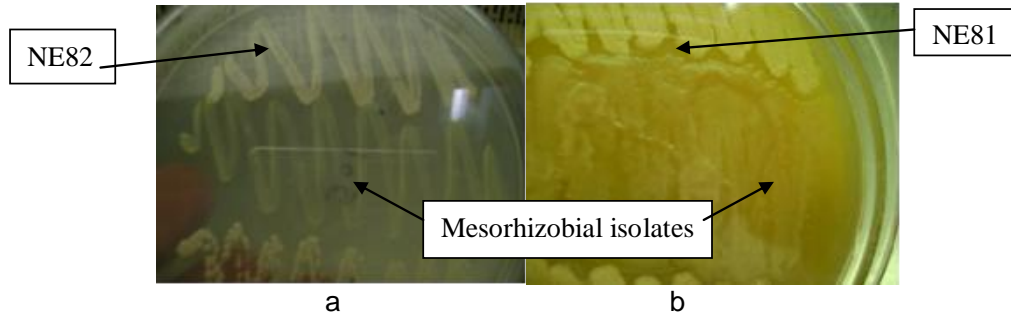
Nodule epiphyte	NE81	NE82	NE1043
NE14	No Effect	No Effect	-
NE16-1	-	No Effect	No Effect
NE16-2	No Effect	No Effect	-
NE18	No Effect	No Effect	-
NE19	No Effect	-	No Effect
NE26	No Effect	-	No Effect
NE32	-	-	No Effect
NE42	No Effect	No Effect	No Effect
NE44-2	-	-	No Effect
NE45	-	No Effect	No Effect
NE53	No Effect	No Effect	-
NE84	-	-	No Effect
NE1006	No Effect	No Effect	-

No effect was observed on the rest twelve rhizospheric bacterial epiphytes.

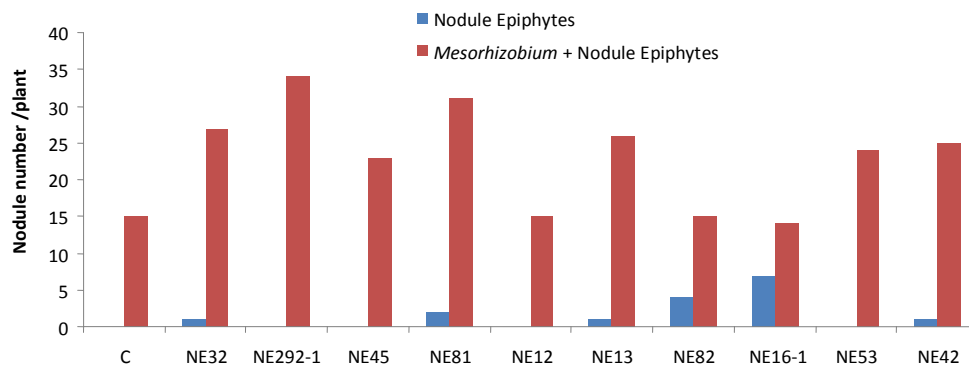


**Figure 5.** Medium components solubilisation by one of the bacterial epiphyte NE81 isolated from the surface of chickpea nodules.

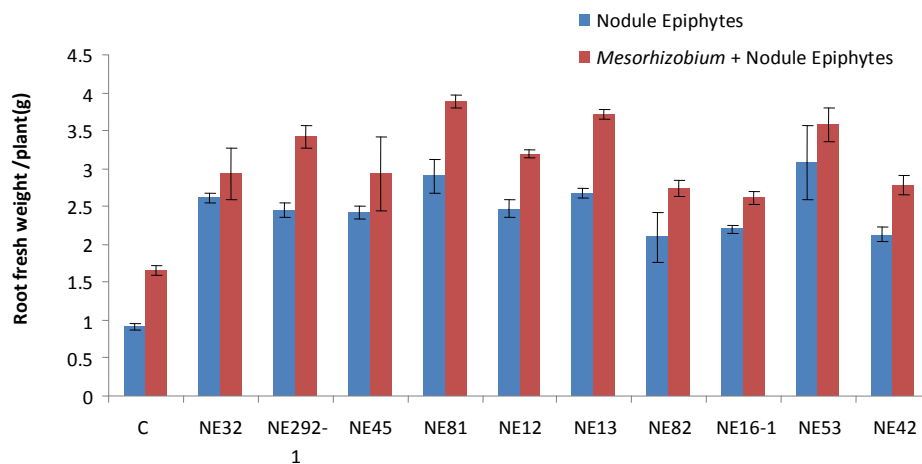




**Figure 6.** Growth promotion activity shown by the nodule epiphytes NE82 and NE81 against mesorhizobial isolates by paired test.



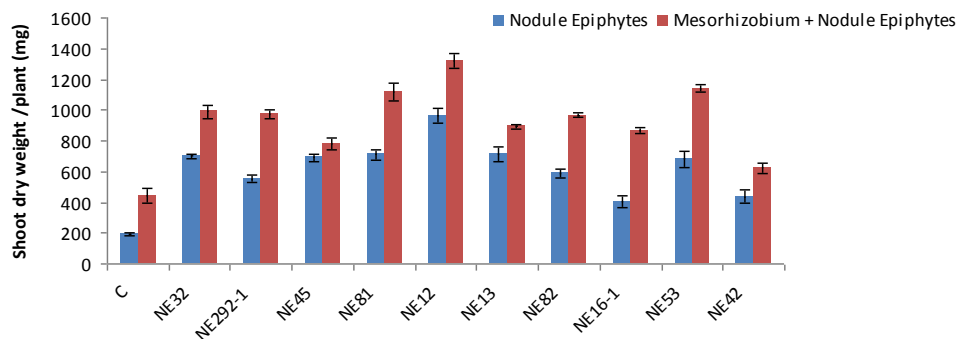
**Figure 7.** Effect of nodule epiphytes together with mesorhizobial inoculation on nodulation of chickpea under sterilized conditions.



**Figure 8.** Effect of nodule epiphytes together with mesorhizobial inoculation on root biomass of chickpea under sterilized conditions.

Similarly, there was also a significant increase in root fresh weight when compared with the control (0.91 g/plant). Root fresh weight was 1.66 g/plant when seeds were inoculated alone with *Mesorhizobium* ( $P < 0.05$ ). It

ranged from 2.1 to 3.07 g/plant in the case of nodule epiphytes from both cultivars and 2.61 to 3.88 g/plant in the case of co-inoculation with nodule epiphytes and mesorhizobial isolate. The highest root fresh weight was



**Figure 9.** Effect of nodule epiphytes together with mesorhizobial inoculation on shoot biomass of chickpea under sterilized conditions.

observed in the case of plants co-inoculated with *Mesorhizobium* and NE81. The significant increase in root fresh weight was observed only when seeds were co-inoculated with NE45 and *Mesorhizobium* ( $P$  value < 0.05) and insignificant when inoculated along with other nodule epiphytes.

## DISCUSSION

Twenty eight nodule epiphytes associated with nodules of the two chickpea cultivars exhibited variation in colony characteristics. A large variation in the colony morphology of isolates from nodules of soybean and *Lespedeza* sp. has also been reported (Hung et al., 2007; Palaniappan et al., 2010). However, majority (93.4%) of the isolates from soybean nodules were Gram positive whereas 84.6% isolates from *Lespedeza* sp. were Gram negative. In the present study, all the isolates identified from the surface of chickpea nodules were Gram positive. This is probably because the geographical location (Hisar), has a wide temperature range (-2 to 47°C) and with these adverse temperature variations, it is quite possible that Gram positive bacteria, and particularly spore formers are the better survivors.

Out of 28 isolates, only three isolates (NE81, NE82 and NE1043) showed zone of inhibition against different isolates. These nodule epiphytes may therefore indirectly help the plants by inhibiting the growth of other harmful microorganisms. Bacteria associated with the surface of roots and nodules have been shown to have beneficial effects on plants. *Bacillus* species has been found to promote the growth of large number of plants and effective in the biological control of many plant microbial diseases (de Freitas et al., 1997; Kokalis-Burelle et al., 2002). In the present study, the isolate NE81 was found to solubilise the media components, suggesting its role in nutrient solubilisation in the soil. Several reports have also indicated that the rhizobacteria promote the plant growth by increasing the availability of nutrients for the plant in the rhizosphere (Glick, 1995; Vessey, 2003). These bacteria help in solubilisation of unavailable forms of nutri-

ents, siderophore production and ammonia production (Stevenson and Cole, 1999).

A significant increase in the nodulation of chickpea plants was observed when they were co-inoculated with *Mesorhizobium* and nodule epiphytes as compared to nodule epiphytes alone. The co-inoculation of NE 292-1 and *Mesorhizobium* led to maximum nodulation whereas *Mesorhizobium* and NE 53 resulted in significant increase in shoot dry weight in chickpea. These observations show that *Mesorhizobium* along with nodule epiphytes have beneficial effect on the plant growth. Similarly, different workers have also reported the use of co-inoculants in nodule stimulation and plant growth promotion of different leguminous crops. A significant increase in nodule weight and shoot biomass in chickpea was reported when co-inoculated with *Mesorhizobium* and *Pseudomonas* (Goel et al., 2000; Sindhu et al., 2002b). The co-inoculation of *Rhizobium* with *Pseudomonas striata* or *Bacillus polymyxa* increased nodulation, nitrogenase activity, nitrogen and phosphate uptake in blackgram (Prasad and Chandra, 2003; Gunasekaran et al., 2004). The inoculation of pigeonpea and mungbean seeds with multiple co-inoculants consisting of *Rhizobium*, *Azotobacter*, *Azospirillum*, *Pseudomonas* and *Bacillus* produced maximum nodule biomass, plant biomass and total soil N in the case of pigeonpea and mungbean hosts (Suneja et al., 2007).

Our study suggests that the nodule epiphytes play a significant role in promoting plant growth either by inhibiting the pathogenic microorganisms or by producing some factors which stimulate the interaction of mesorhizobia with plant roots. These can further be used as co-inoculants together with *Mesorhizobium* to enhance the plant growth. However, their effectiveness on the crop should be assessed under field conditions also.

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*Full Length Research Paper*

## Role of QuantiFERON-TB Gold In-Tube test in detection of latent tuberculosis infection in health care contacts in Alexandria, Egypt

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Tuberculosis infection represents a global health problem and a great risk to health care workers. The detection and treatment of latent tuberculosis infection is a key strategy in the control of tuberculosis. The aim of this study was to estimate the usefulness of QuantiFERON-TB Gold In-Tube test which is an interferon-gamma release assay for the detection of latent tuberculosis infection, in a group of Egyptian Health Care Workers in comparison to the tuberculin skin test. A total of 100 Health Care Workers were enrolled. Subjects diagnosed as latent tuberculosis infection by tuberculin were 88% as compared to 36% by quantiferon. Higher tuberculin positive results were associated with direct contact to tuberculosis patients and job categories as physicians and nurses, while higher quantiferon positive results were associated with longer duration of employment and absence of BCG vaccination. The overall agreement between the two tests was poor ( $k=0.109$ ). The negative discordant results (quantiferon negative/tuberculin positive) were 53% and were found among both contacts and non contact group, which could reflect high number of false positive tuberculin skin test. These data suggest that using IFN- $\gamma$  method to screen new entrant health care workers for latent tuberculosis infection in our population could be more helpful because it is more specific for detecting latent tuberculosis infection compared to tuberculin skin test.

**Key words:** Tuberculin, gamma-interferon, health care, latent infection.

### INTRODUCTION

Occupational latent tuberculosis infection (LTBI) among health care workers (HCWs) is an important public health issue (Adachi et al., 2013). HCW is anyone working in a healthcare setting, regardless of direct patient TB contact (Dorman et al., 2014). Recent systematic analysis showed

that the risk for tuberculosis (TB) among HCWs is consistently higher than the risk among the general population worldwide, irrespective of TB incidence in each country, and confirmed that TB is an occupational disease (Baussano et al., 2011).

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TB prevalence rate in Egypt was estimated in the World Health Organization (WHO) global report (2013) to be 29 per 100,000 populations. TB transmission in healthcare facilities can be significantly reduced with the implementation of effective TB infection control measures. While most high income countries have successfully implemented TB infection control measures, TB infection control measures are limited or virtually non-existent in most resource-limited countries (Whitaker et al., 2013).

Most high-income countries screen HCWs periodically for LTBI as part of their TB infection control programs (Whitaker et al., 2013), in an effort to identify new infections that can be targeted for preventive therapy (Zwerling et al., 2012a), but this practice is unusual in most low and middle-income countries (Whitaker et al., 2013).

Although several tests have been used to diagnose LTBI, yet till now there is no gold-standard test for the diagnosis (Dheda et al., 2005; Francis et al., 2007; Pai et al., 2007). Traditionally TB screening in HCWs has been conducted using the Tuberculin Skin Test (TST), a test with known limitations. Recently, Interferon gamma release assays (IGRAs) are being increasingly used for LTBI screening. Two commercially available IGRAs have been approved for use: the Quanti-FERON-TB Gold In-Tube (QFT-GIT) assay (Cellestis Inc., Valencia CA) and the T-SPOT.TB assay (Oxford Immunotec, Abingdon, UK). IGRAs have several advantages over the TST: they require only one visit, are not affected by BCG vaccination, have less cross-reaction with non-tuberculous mycobacteria, are less subjective in measuring results, and can be repeated without boosting (Whitaker et al., 2013). In comparison with TST, the IGRA reduces the number of x-rays and the amount of chemoprevention needed (Nienhaus et al., 2013). Yet, some limitations have been reported such as false-negative or indeterminate results caused by incorrect blood sampling, improper handling of the specimen which may limit exposure of lymphocytes to the presenting antigen, or specimen obtained prior to the development of cellular immune response. Moreover, heterophile antibodies formed during other inflammatory conditions may interfere with specific responses to ESAT-6, CFP-10, or TB7.7 (p4) peptides (Mazurek et al., 2005).

Several systematic reviews have suggested that IGRAs are as sensitive, and as more specific than the TST in identifying LTBI, particularly in low TB incidence settings (Zwerling et al., 2012a). However, in high-incidence settings, there were no consistent differences in the prevalence of positive tests (Zwerling et al., 2012b). A WHO policy statement on the use of IGRA in low- and middle-income countries indicates that "data on serial testing and reproducibility of IGRAs, as well as evidence on the predictive value of IGRAs in HCWs, are still absent for high – incidence settings" (WHO, 2011).

Although the comparison of TST and QFT-GIT has been shown in the literature extensively, limited data are available concerning the study of both tests among HCWs population in Egypt (medium/high TB setting). The

aim of the present study was to evaluate the usefulness of the QFT-GIT test for detecting LTBI among a group of Egyptian HCWs, in comparison to the TST.

## MATERIALS AND METHODS

This study was carried out over a 3 month period from June through August 2010, and it consisted of 2 groups: 80 HCWs, selected from employees in EL-Maamora Chest Hospital TB departments, who had a close contact to sputum smear positive pulmonary TB patients for at least 3 months duration (contact group) (Morsy et al., 1997), and 20 non-exposed individuals from staff working in administrative sections that have no contact with TB patients, at Alexandria Main University Hospital (AMUH) as a control group. An informed consent was taken from all participants in the study. First, 3 ml blood was collected from each HCW for the QFT-GIT test, which was performed at the microbiology laboratory department at AMUH. Next, TST was performed for all HCWs at their work place. The study was approved by the ethics committee of AMUH.

HCWs completed a questionnaire covering demographic data, occupational and non-occupational TB exposure in the occupational environment and the duration of contact with TB patients, BCG vaccination status, history of former active TB infection, previous treatment for TB, results of previous TST, work history in the health care institution, potential non-occupational exposure to TB. BCG vaccination status is verified by scars.

### Tuberculin skin test (TST)

The TST was performed using the Mantoux method by a trained nurse according to manufacturer instructions; 5TU (0.1 ml of Tuberculin PPD (Vacsera, Giza, Egypt) were injected intradermally into the inner aspect of the forearm. Correct injection was indicated by producing a palpable elevation of the skin (wheel) 6-10 mm in diameter. The test was read 48-72 h. Interpretation of the results was done according to American Thoracic Society guidelines (2000), positive test is indicated by the presence of palpable induration of 10 mm or more.

### QuantIFERON®-TB Gold In-Tube test (QFT-GIT)

The QuantiFERON-TB Gold In-Tube test (QFT-GIT) (Cellestis Ltd/Qiagen, Carnegie, Australia) was performed as per the manufacturer's instructions. The system uses two specialized blood collection tubes; one of them contains antigens representing certain *M. tuberculosis* proteins ESAT- 6, CFP-10, and TB-7.7 (p4) as well as negative (Nil) controls. One mL of blood by venipuncture was introduced directly into each of the collection tubes. The tubes were vigorously shaken for 5 seconds (or 10 times) till frothing to ensure that the entire inner surface of the tube has been coated with blood. The tubes were incubated at 37°C incubator as soon as possible, and within 16 h of collection. Incubation of the blood occurs in the tubes for 16 to 24 h, after which plasma is harvested by centrifugation and tested for the presence of IFN- $\gamma$  by enzyme-linked immunosorbent assay (ELISA). The Optical Density (OD) of each well was measured within 1 hour of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 to 650 nm reference filter. The cut-off value for positive results was  $\geq 0.35$  IU/ml. version 20. Categorical variables were described using frequencies and percentages. Chi square, Fisher's exact; McNemar and Monte Carlo tests were used for testing associations between categorical variables. Agreement test; reported as a kappa statistic, was used as a quantitative measure of the strength of agreement between tests, where  $K < 0.20$  (poor agreement),  $K = 0.21 - 0.40$  (fair agreement),  $K = 0.41 - 0.60$  (moderate agreement),  $K = 0.61 -$

**Table 1.** Participant characteristics (n=100).

Characteristic	n (%)
<b>Gender</b>	
Male	14(14)
Female	86(86)
<b>Age</b>	
20- 29	35(35)
30-39	28(28)
≥ 40	37(37)
<b>Occupation</b>	
Nurses	64(64)
Clinician	21(21)
Worker (housekeeping)	8(8)
Lab Technician	7(7)
<b>BCG Vaccination</b>	
Yes	83(83)
No	17(17)
<b>Direct Contact of TB Patients</b>	
Yes	80(80)
No	20(20)
<b>Duration of Work (years)</b>	
≤ 1	4(4)
2-5	28(28)
6-9	23(23)
≥ 10	45(45)

0.80 (good agreement),  $K=0.81 - 1.00$  (very good agreement). P value of kappa was also reported. Non-parametric statistical tests of significance were applied to ranks; Mann-Whitney test was used to compare two independent groups. Statistical significance was accepted as  $p < 0.05$ . All applied statistical tests of significance were two-tailed.

## RESULTS

### Participant demographics

Participant characteristics are displayed in Table 1. The mean age of the participants was  $36.56 \pm 9.58$  yrs. The majority was females (86%) and a history of BCG vaccination was recorded in 83% of the participants. 64% of the study population were nurses, 21% clinicians 7% technicians and 8% housekeepers. 80% of the participants had direct contact with TB patients, with mean length of employment duration as a HCW in the institution was  $10.55 \pm 7.69$  yrs.

### Diagnostic tests for latent TB

As shown in Table 2, a total of 88 HCWs had a positive TST using a cut off  $\geq 10$  mm induration. In univariate ana-

lysis, working as a nurse and direct exposure to infectious TB patients were significantly associated with having a positive TST result. Female gender, age  $> 40$  years, BCG vaccination and duration of employment in HCW institution  $\geq 10$  years were associated with higher percentage of TST positivity, but the differences were statistically non significant.

The prevalence of LTBI among the 100 participants, assessed by QFT-GIT was 36% and it correlated with the absence of BCG vaccination (58.8%) ( $p= 0.03$ ) and a duration of employment as HCW  $\geq 10$  years (48.9%) ( $p = 0.014$ ). No statistically significant association was observed for gender, age, job category and direct exposure to TB patients, despite higher rates of QFT-GIT positivity among males, working as clinician or nurse and age  $\geq 40$  years.

### Test concordance and discordance

Table 3 shows that the concordant results (46%), predominantly positive (35%), were significantly associated with clinicians ( $MCp= 0.014$ ). The discordant results were observed in 54 HCWs, most of them (53%) had positive TST/ negative QFT-GIT combination, which was significantly associated with nurse occupation ( $MCp= 0.014$ ).

Concordant and discordant results were not significantly associated with age, gender, previous BCG vaccination, direct contact with TB patients and duration of exposure to TB patients ( $P>0.05$ ). Positive concordant results increased among males, age  $\geq 40$  years, absence of BCG vaccination, duration of employment in HC institution  $\geq 10$  years, while most females, HCWs younger than 30 years, history of BCG vaccination, direct contact with TB patients showed higher rates of negative discordant results.

### Test agreement

Table 4 shows a significant poor overall agreement between both tests among all HCWs (Kappa = 0.109,  $p = 0.033$ , 95% CI = 0.023, 0.195). Among BCG vaccinated HCWs, significant poor agreement was also observed. (Kappa = 0.105,  $p = 0.032$ , 95% CI = 0.032, 0.178). Concordance and discordance between results of both tests were not statistically significant among the 17 non BCG vaccinated HCWs. Among the 80 contacts HCWs, TST results were constantly positive, so no statistics could be computed. Also, it was observed that high negative discordant results (QFT-/TST+) were observed among both contact and control groups (57.5% and 35% respectively).

The limitations of this study: 1. Low number of patients. 2. Lack of a gold standard for LTBI. 3. Cross-sectional design with no longitudinal follow up. 4. There is a possibility that some people, who do not describe an exposure to patients with TB, may have an inadvertent exposure.

**Table 2.** Results of univariate analyses of potential risk factors for detection of LTBI by means of TST and QFT-GIT among the 100 HCWs

Characteristic	N (100)	TST		Test statistic (P value)	QFT-GIT		Test statistic (P value)
		Positive n (%)	Negative n (%)		Positive n (%)	Negative n (%)	
<b>Gender</b>							
Male	14	11 (78.6)	3 (21.4)	<i>(FEp= 0.367)</i>	8 (57.1)	6 (42.9)	$\chi^2= 3.158$ (0.076)
Female	86	77 (89.5)	9 (10.5)		28 (32.6)	58 (67.4)	
<b>Age</b>							
20-29	35	32 (91.4)	3 (8.6)	$Z= 0.265$ (0.791)	9 (25.7)	26 (74.3)	$Z= 1.779$ (0.075)
30-39	28	23 (82.1)	5 (17.9)		10 (35.7)	18 (64.3)	
≥ 40	37	33 (89.2)	4 (10.8)		17 (45.9)	20 (54.1)	
<b>Occupation*</b>							
Nurse	64	64 (100)	0 (0)	<i>MCp &lt;0.001</i>	22 (34.4)	42 (65.6)	<i>(MCp = 0.244)</i>
Clinician	21	17 (81)	4 (19)		11 (52.4)	10 (47.6)	
Worker (housekeeping)	8	5 (62.5)	3 (37.5)		2 (25)	6 (75)	
Lab Technician	7	2 (28.6)	5 (71.4)		1 (14.3)	6 (85.7)	
<b>BCG Vaccination</b>							
Yes	83	74 (89.2)	9 (10.8)	<i>(FEp=0.424)</i>	26 (31.3)	57 (68.7)	$\chi^2= 4.631$ (0.031)
No	17	14 (82.4)	3 (17.6)		10 (58.8)	7 (41.2)	
<b>Direct Contact with TB patients</b>							
Yes	80	80 (100)	0 (0)	<i>(FEp &lt;0.001)</i>	34 (42.5)	46 (57.5)	$\chi^2= 7.335$ (0.077)
No	20	8 (40)	12(60)		2 (10)	18 (90)	
<b>Duration of Work (years)</b>							
≤ 1	4	4 (100)	0 (0)	$Z=1.752$ (0.080)	1 (25)	3 (75)	$Z= 2.453$ (0.014)
2-5	28	21 (75)	7 (25)		6 (21.4)	22 (78.6)	
6-9	23	21 (91.3)	2 (8.7)		7 (30.4)	16 (69.6)	
≥ 10	45	42 (93.3)	3 (6.7)		22 (48.9)	23 (51.1)	

**Table 3.** Distribution of the concordant and discordant results for QFT-GIT and TST tests.

Characteristic	N	Concordant (n = 46)		Discordant (n = 54)		Test statistic (P value)
		Neg/Neg (n=11) n (%)	Pos/Pos (n=35) n (%)	Pos <sup>a</sup> /Neg (n=1) n (%)	Neg <sup>b</sup> /Pos (n=53) n (%)	
<b>Gender</b>						
Male	14	2 (14.2)	7 (50)	1 (7.14)	4 (28.5)	$\chi^2= 2.191$ (0.139)
Female	86	9 (10.5)	28 (32.5)	0 (0)	49 (56.9)	
<b>Age</b>						
20-29	35	3 (8.57)	9 (25.7)	0 (0)	23 (67.7)	$Z= 1.423$ (0.155)
30-39	28	5 (17.8)	10 (35.7)	0 (0)	13 (46.4)	
≥40	37	3 (8.1)	16 (43.2)	1 (2.7)	17 (45.9)	
20-29	35	3 (8.57)	9 (25.7)	0 (0)	23 (67.7)	$Z= 1.423$ (0.155)
30-39	28	5 (17.8)	10 (35.7)	0 (0)	13 (46.4)	
≥40	37	3 (8.1)	16 (43.2)	1 (2.7)	17 (45.9)	

Table 3. Contd.

<b>Occupation</b>						
Nurses	64	0 (0)	22 (34.4)	0 (0)	42 (65.6)	<i>(MCp= 0.014)</i>
Clinician	21	4 (19.1)	11 (52.4)	0 (0)	6 (28.6)	
Worker (housekeeping)	8	3 (37.5)	2 (25)	0 (0)	3 (37.5)	
Lab Technician	7	4 (57.1)	0 (0)	1 (14.3)	2 (28.6)	
<b>BCG Vaccination</b>						
Yes	83	9 (10.8)	26 (31.3)	0 (0)	48 (57.8)	$\chi^2=2.885$ (0.089)
No	17	2 (11.8)	9 (52.9)	1 (5.9)	5 (29.4)	
<b>Direct Contact of TB Patients</b>						
Yes	80	0 (0)	34 (42.5)	0 (0)	46 (57.5)	$\chi^2=1.973$ (0.160)
No	20	11 (55)	1 (5)	1 (5)	7 (35)	
<b>Duration of Work (years)</b>						
≤ 1	4	0 (0)	1 (25)	0 (0)	3 (75)	$Z= 0.813$ (0.416)
1-5	28	7(25)	6 (21.4)	0 (0)	15 (53.6)	
6-9	23	2 (8.7)	7 (30.4)	0 (0)	14 (60.9)	
≥ 10	45	2 (4.4)	21(46.7)	1 (2.2)	21 (46.7)	
≤ 1	4	0 (0)	1 (25)	0 (0)	3 (75)	$Z= 0.813$ (0.416)
1-5	28	7(25)	6 (21.4)	0 (0)	15 (53.6)	
6-9	23	2 (8.7)	7 (30.4)	0 (0)	14 (60.9)	
≥ 10	45	2 (4.4)	21(46.7)	1 (2.2)	21 (46.7)	

<sup>a</sup>Positive by QFT-GIT, <sup>b</sup>Negative by QFT-GIT

Table 4. Agreement and disagreement between results of QFT-GIT and TST and effect of BCG vaccination and contact with TB patients.

Parameter	N	PCR	NCR	Kappa	p	DR <sup>a</sup>	NDR <sup>b</sup>	McNemar	
		n (%)	n (%)	Value		95% CI	n (%)	n (%)	p
All subjects	100	35 (35.0)	11 (11.0)	0.109	0.023, 0.195	0.033	1 (1.0)	53 (53.0)	<0.001
BCG vaccinated	83	26 (31.3)	9 (10.8)	0.105	0.032, 0.178	0.032	0 (0.0)	48 (57.8)	<0.001
Not BCG vaccinated	17	9 (52.9)	2 (11.8)	0.203	-0.213, 0.619	0.323	1 (5.9)	5 (29.4)	0.219
Contacts	80	34 (42.5)	-	-	-	-	-	46 (57.5)	-
Non- contacts	20	1 (5.0)	11 (55.0)	0.048	-0.0269, 0.366	0.761	1 (5.0)	7 (35.0)	0.070

PCR; positive concordance rate, NCR; negative concordance rate, PDR; positive discordance rate, NDR; negative discordance rate. <sup>a</sup>Positive by QFT-GIT, <sup>b</sup>Negative by QFT-GIT.

## DISCUSSION

In our study, the proportion of LTBI assessed by TST was 88 and 36% by QFT-GIT. Close results were reported in Egypt in Abu-Taleb et al. study (2011), where 71.1% of contacts were diagnosed as latent TB infection by TST and 31% by IFN- $\gamma$  assay. This discrepancy might be explained by high false positives TST results, as HCWs repeat TSTs during their employment, which may further boost their reaction to the TST. This hypothesis is supported by Kang et al. (2005) study.

Our TST results were highest rate among nurses (100%) and clinicians (81%). This was in agreement with Drobniowski et al. (2007) study, where the proportion of LTBI assessed by TST was 40.8% and was significantly higher in doctors and nurses (39.1%). Significant positive TST results were higher among our HCWs in direct contact with TB patients. This was in accordance with Mirtskhulava et al. (2008) and Machado et al.,(2009) studies, where TST results in HCWs and household contacts with TB patients were (67% and 55.6% respectively). Lower result (49.5%) was reported in the study of



Helmy et al. (2011), conducted on a population of close household TB contacts in Egypt. Even very low results were reported by other studies, such as 12.8% (Nienhaus et al., 2008a) and 7.2% (Schablon et al., 2009) in Germany. This could be explained by the low prevalence of TB in Germany in comparison with Egypt and Brazil.

In the present study, there was no statistically significant association between positive TST results and gender, age and duration of employment in the health care institution. This may be explained by the small number of the study group. The rate of detection of LTBI assessed by QFT-GIT in our study was 36%. Low detection rates by QFT-GIT as compared to TST were also reported by other scientists: Harada et al. (2006), Soborg et al. (2007), Niehaus et al. (2008a), Stebler et al. (2008) and Ringshausen et al., (2009).

But the relatively high results of positive QFT-GIT found in HCWs (36%) in this study may reflect the higher infection risk in this occupational area due to intermediate prevalence of TB in our country (29 per 100000 populations) as what was reported by Khanna et al. (2009), who stated that participants who were born in a country with a high TB prevalence, and/or who were born in Africa were significantly more likely to have a positive QFT-GIT result. Higher QFT-GIT results was reported by Helmy et al. (2011), in Egypt, being 49.5% in a population of close household TB contacts, which may be explained by the intimate closer contact between the TB patients and the close household contacts.

The small sample size in this study might explain the reported insignificant correlation between positive QFT-GIT results and occupation. Other studies investigating the risk of physicians did not indicate an increased infection risk for physicians (Schablon et al., 2009). Other researchers reported a prevalence of LTBI in nurses; a subgroup with high level of patient contact, and thus potential exposure to TB cases (Joshi et al., 2006; Yanai et al., 2003; Garcia-Garcia et al., 2001).

There was a significant positive results of QFT-GIT associated with longer duration of work ( $\geq 6$  years). The rate of detection of LTBI assessed by QFT-GIT was significantly higher in non-BCG vaccinated than in vaccinated HCWs. This indicates that QFT-GIT is not affected by previous BCG vaccination as it utilizes *M. tuberculosis* specific antigens (Nienhaus et al., 2011; Andersen et al., 2000).

Our QFT-GIT positive results were higher in older age group ( $\geq 40$  years), a finding that was supported by Ringshausen et al. (2009), which might indicate age dependency of QFT-GIT that is not observed with TST. Moreover, QFT-GIT positive results were higher among HCWs in direct contact with TB patients, as what was reported in Stebler et al. (2008) and Mirskhulava et al. (2008) studies.

In this study, concordant results were lower than discordant results (46 and 54% of HCWs, respectively). The results of the present study were matching with

Harada et al. (2006) who compared the performance of TST and Quantiferon in a total of 304 HCWs. They found that concordant results were lower than discordant results (14 and 86%, respectively).

On the other hand, the present results disagreed with the Egyptian study of Helmy et al. (2011), where concordant results were (65.5%) and discordant results (34.4%) and were not affected by gender or age. Other non matching studies: Connell et al. (2006) and Kang et al. (2005), where the concordant results were higher than the discordant results.

Positive concordant results (QFT+/TST+) increased with increasing age and increasing duration of work as HCW in this study. Joshi et al. (2006) reported that increasing age and duration of employment in health care facility (indicating longer cumulative exposure) were associated with higher prevalence of LTBI in most studies.

The QFT-/TST+ discordance increased from 29.4% in non BCG vaccinated to 57.8% in vaccinated HCWs, and most of non-vaccinated had concordant positive results. This was in accordance with Nienhaus et al. (2011) study, which reflects that TST is usually affected by previous BCG vaccination, while QFT-GIT is not affected and hence low specificity of TST in detection of LTBI in comparison with QFT-GIT.

Brock et al. (2004) compared the performance of TST and Quantiferon in a total of 45 contacts of persons having active pulmonary TB, none of them was BCG vaccinated. Concordant results were obtained in 93% of cases while in only 7% discordant results were obtained. The higher concordance here may be attributed to the absence of BCG vaccination and this confirms the concept that BCG vaccination affects greatly performance of TST. In 3 studies of Kang et al. (2005), Harada et al. (2006) and Diel et al. (2006a) discordance was greater in persons with BCG vaccination than in those who were not vaccinated.

The poor overall agreement ( $k= 0.109$ ) reported between TST and QFT-GIT tests among our HCWs, may be explained by the negative discordant QFT -/ TST+ results found in more than half the subjects (53%). Poor agreement was also reported by other studies (Ringshausen et al., 2009; Vinton et al., 2009; Nienhaus et al., 2008b; Diel et al., 2006b) and all confirmed that the BCG is a major confounder of TST results. These negative discordant results were observed among both contact and non-contact group (57.5% and 35% respectively). This could reflect a high rate of false positive TST, which could be explained by exposure to non tuberculous mycobacteria (Franken et al., 2007), or the fact that the antigen used in TST (PPD) is a shared antigen present in *M. tuberculosis* complex and non-tuberculous mycobacteria (Pai et al., 2004; Menzies et al., 2007), but in QFT-GIT the antigens used are *M. tuberculosis* specific antigens therefore considered better than TST in screening of HCWs for LTBI (Brook et al., 2004).

## CONCLUSION AND RECOMMENDATION

Results of LTBI detected by the TST were higher than those detected by QFT-GIT among our group of Egyptian HCWs. Higher TST positive results were associated with direct contact to TB patients and certain job categories as physicians and nurses, while higher QFT-GIT positive results were associated with longer duration of employment and absence of BCG vaccination. The poor overall agreement ( $k=0.109$ ) and the high negative discordant results (QFT-/TST+) can be explained by high BCG vaccination coverage in our country. The rate of discordant results (QFT-/TST+) were found among both contacts and non contacts group, which again could reflect high number of false positive TST. Therefore, we recommend using QFT-GIT to screen new entrant HCWs for LTBI in our population because it is more specific as compared to TST. Also, QFT-GIT may provide additional information for diagnosis and strategic management of preventive treatment in BCG vaccinated HCWs, avoiding unnecessary treatment to those not really infected.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

## Antibiotic resistant pattern of isolated bacteria from Obere River in Orile-Igbon, Oyo State, Nigeria

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Good quality water must be odourless, colourless, tasteless and free from pathogens. This is adversely affected when it is polluted beyond certain limits. Potable water for domestic use should however be free of pathogenic organisms. Water samples were collected from Obere River in Orile-Igbon, Oyo state, Nigeria in order to study the antibiotic resistance pattern of isolated bacteria. The bacteria genera detected were *Pseudomonas*, *Bacillus*, *Proteus* and *Flavobacterium*. The isolates were subjected to antibiotic sensitivity test with about 90% showing multiple antibiotic resistance. All the isolates have a high resistance rate (100%) against amoxicillin, augmentin (100%), streptomycin (95%), chloramphenicol (95%) and ceftriaxone (90%); while, the least resisted antibiotics was ciprofloxacin (35%). It was concluded that Obere River in Orile-Igbon, Oyo State is polluted with pathogenic bacteria showing multiple antibiotic resistance. Thus, there is need for proper treatment of water in this study area before distribution for consumption and general domestic usage.

**Key words:** Water samples, Obere River, antibiotic sensitivity test, multiple antibiotic resistance.

### INTRODUCTION

In the present era, man has made flowing water a medium for getting rid of his wastes from anthropogenic sources thus introducing noxious substances into them consequently polluting the water bodies. These wastes adversely affect the physico-chemical properties as well as the microbial component of the water bodies which then affect lives in it. Obere River is located in Orile igbon, Ogbomoso, Oyo State, Nigeria, with poultry farm, cement industry, market, cassava processing sites and arable crop farms located along its course. The river serves as a source of water for nearby dwellers; it is used for drinking, cooking, swimming, etc.

Contaminated water bodies often serve as natural habitats of pathogenic coliforms, thereby playing a role in disease process (Ademola et al., 2009). Antibiotic resistance then sets in as a result of insurgent sub-therapeutic concentration of antibiotics in wastewater

discharged into natural water bodies (Diwan et al., 2010). In developing countries, high population growth has led to increased human activities. These activities have cumulated to indiscriminate dumping of refuse, waste disposal, etc (Akubugwo and Duru, 2011) in water bodies hence, making accessibility and availability of clean and uncontaminated water difficult (Oladipo et al., 2011; Ogbonna et al., 2011).

Studies have been done by various researchers to measure chemical and microbial contaminants in ground water and surface water such as streams, lakes and rivers. Chukwu (2008) reported on the ground water pollution from abattoir waste in Minna state. Garba et al. (2010) reported a mean arsenic concentration of 0.34 mg/l in drinking water from hand dug wells, boreholes and taps of Karaye Local Government area, Kano state. Olaoye and Onilude (2009) have documented varying

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levels of microbial contaminations in drinking water from western parts of Nigeria. Indeed, the microbial quality of potable water should not exceed the limits specified in the water quality guideline (APHA, 1998). Before the abusive antimicrobial usage, only a slight resistance level had been detected among enteric bacterial pathogens. Nowadays susceptibility to antimicrobials has changed and resistant patterns have been used as epidemiological markers (Bechtluft et al., 2008). The indiscriminate usage and subsequent release of residual antibiotics in wastewater is considered an important factor for the emergence, selection and dissemination of antimicrobial resistant bacteria. Drinking water must meet specific criteria and standards to ensure that water supplied to the public is safe and free of pathogenic micro organisms as well as hazardous compounds. Therefore, this research work is meant to microbiologically analyze Obere River which is consumed by Orile-Igbon residents in Oyo State and determine if its portability is justified.

## MATERIALS AND METHODS

### Sample collection

Water samples were aseptically collected from Obere River, Orile-Igbo, Ogbomoso, Oyo state. For the purpose of this study, the river was divided into three sampling points. Samples were collected into 250 ml sterile plastic container from the three sampling points between 9:00 am and 10:00 am. The samples were collected once in a month for a period of three months.

### Media preparation

Nutrient Agar powder weighing 14 g was dispensed in 500 ml of distilled water in conical flask. After mixing the solution, it was heated gently to dissolve and then autoclaved. The agar was allowed to cool to about 45°C then poured into sterile disposable Petri dishes.

### Isolation of microorganisms

Serial dilution was carried out on the samples and then inoculated unto nutrient agar plate using the spread plate method. The plates were incubated at 35°C for 24 h. The colonies observed on the plates were then subcultured and stored on slants at 4°C for further use.

### Identification of isolated micro organisms

Biochemical and morphological characterization of the isolates were done using the manual of Berger and Petz (1991).

### Antibiotic sensitivity test

The *in vitro* antibiotic susceptibility test of the bacterial isolates was performed using the standardized disc diffusion method described by Bauer et al. (1996). Sterile Petri dishes of Mueller Hinton agar were prepared. 0.1 ml of each organism was then seeded into Mueller-Hinton agar plates and allowed to stand for 45 min. It was

**Table 1.** Distribution and proportion of bacteria in Obere River.

Isolates identified	Prevalence
<i>Pseudomonas</i> spp.	10 (50%)
<i>Proteus</i> spp.	4 (20%)
<i>Flavobacterium</i> spp.	4 (20%)
<i>Bacillus</i> spp.	2 (10%)

investigated using Fonz laboratory antibiotic sensitivity disc for both Gram positive and Gram negative discs containing: Augmentin- 30 µg, Amoxicillin - 25 µg, Erythromycin- 5 µg, Tetracycline- 30 µg, Nitrofurantoin- 200 µg, Gentamycin-10 µg Cotrimoxazole- 25 µg, Oxofloxacin- 5 µg, Pefloxacin- 5 µg, Amoxicillin- 25 µg, Chloramphenicol-30 µg, Ceftriaxone-30µg, Gentamycin-10 µg, Pefloxacin-5 µg, Cotrimoxazole- 25 µg, Ciprofloxacin-30 µg, Erythromycin- 3 µg. The commercial antibiotic discs were placed on the prepared plates previously seeded with 18 hours broth culture of the test organisms. The plates were incubated at 37°C, 48 h after which the zones of inhibition were measured in millimeters and interpreted accordingly considering the appropriate break point (Andrew, 2008).

## RESULTS

A total number of 20 bacterial isolates belonging to 4 genera were obtained. *Pseudomonas*, *Flavobacterium*, *Bacillus* and *Proteus*, their distribution are as shown in Table 1. A high occurrence was obtained in *Pseudomonas* species 10 (50 %), followed by *Proteus* species 4 (20%), *Flavobacterium* species 4 (20%) and *Bacillus* species 2 (10%). The number of antibiotics to which resistance was shown is as presented in Table 2, while Figure 1 reflects the percentage resistance of the isolates to each antibiotics. Augmentin and Amoxicillin was 100% resisted, with Ciprofloxacin being the least resisted antibiotics.

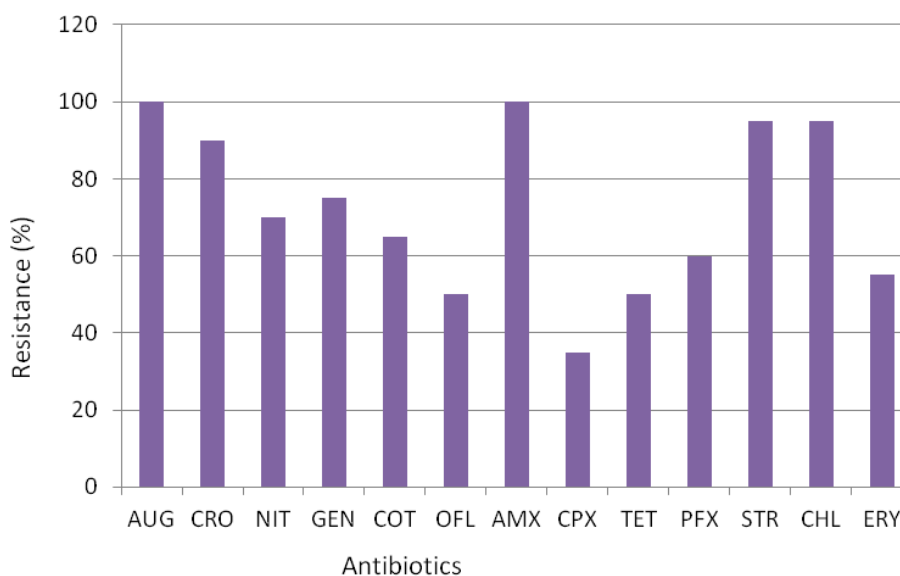
Table 3 presents the antibiotic resistance profile for all the isolates. *Pseudomonas* and *Bacillus* species exhibited the highest number of resistance against the antibiotics.

## DISCUSSION

All the isolates have a high resistance rate (100%) against Amoxicillin, Augmentin (100%), Streptomycin (95%), Chloramphenicol (95%) and Ceftriaxone (90 %). Moreover, from the antibiotic sensitivity profiles in Table 3, each bacterium showed resistance to minimum of seven antibiotics, indicating the multiple resistance pattern characteristic of the isolated bacterial. This could probably result from a recent contamination of the water with sewage; this is a submission reached by Ajayi and Akonai (2005). Adebayo et al. (2012) also reported 96% resistance to Augmentin by most isolates from salad.

**Table 2.** Distribution of antibiotic resistance among bacterial isolates.

Isolates identified	Total number	Number of resistance exhibited to antibiotics												
		AUG	CRO	NIT	GEN	COT	OFL	AMX	CPX	TET	PFX	STR	CHL	ERY
<i>Pseudomonas</i> species	10	10	9	6	8	7	6	10	4	5	5	10	10	7
<i>Proteus</i> species	4	4	4	2	2	2	2	4	0	2	0	4	4	2
<i>Flavobacterium</i> species	4	4	4	4	4	2	0	4	2	2	2	4	4	2
<i>Bacillus</i> species	2	2	1	2	1	2	2	2	2	1	1	1	1	1



**Figure 1.** Antibiotics resistance pattern in percentage. AUG: Augmentin, NIT: nitrofurantoin, OFL= ofloxacin; TET: tetracycline, CPX: ciprofloxacin AMX: amoxicillin, STR: streptomycin; CHL: chloramphenicol; CRO: ceftriaxone, GEN: gentamycin, PFX: pefloxacin, COT: cotrimazole, CPX: ciprofloxacin, ERY: erythromycin.

This implies that either these isolates have been able to circumvent the active ingredient in these antibiotics or probably fake drugs are in circulation. One of the ways to prevent antibiotic resistance of pathogenic species is to use new compounds that are not based on existing antimicrobial agents (Adebayo and Adegoke, 2008; Akinjogunla et al., 2009a).

As shown in this study, the isolates showing resistance to the greatest number of antibiotics was identified in *Bacillus* and *Pseudomonas* strains. This result correlates with the work of Foti et al. (2009) where it was concluded that the highest rate of multiple resistant to antibiotics by Gram negative isolates from the Mediterranean water was found in *Pseudomonas* strains where antibiotics such as Amoxicillin, Tetracycline and Gentamicin were used. Al-Bahry et al. (2009) reported that the source of multiple antibiotic resistant bacteria could be from polluted effluents. Suman et al. (2013) revealed that antibiotic resistance and metal tolerance traits are conjugative plasmid borne, as they could be transferred to sensitive strains through the conjugation process. The contamination of Obere River with antibiotic

resistant bacteria could be as a result of contamination with sewage or effluents from various sources such as poultry farm, cement industry, market, cassava processing sites and arable crop farm.

## Conclusion

Obere River is obviously polluted with antibiotic resistant microorganisms, as shown in this research. The highest multiple antibiotic resistant was mainly demonstrated by *Bacillus* spp. and *Pseudomonas* spp. Most bacterial isolates demonstrated resistance to most antibiotics tested, but the least resistance was observed with ciprofloxacin (35%).

Multiple antibiotic resistance is significant health wise, thus, there is need for proper treatment of water in this study area before distribution for consumption and general domestic usage. Authorities concerned should therefore increase surveillance of water bodies, introduction of new multi-active antibiotics and eradication of fake drugs from circulation.

**Table 3.** Antibiotic resistance profile of bacterial isolates.

Isolate	Number of isolates that demonstrated resistance	Antibiotic resistance pattern	Number of antibiotics resisted
<i>Pseudomonas</i> species	2	AUG, AMX, CRO, STR, CHL, GEN, ERY, COT,	8
	1	AUG, AMX, CRO, STR, OFL, CPX, CHL, GEN, PFX, COT, ERY, NIT, TET	13
	1	AUG, AMX, CRO, STR, OFL, CHL, GEN	7
	2	AUG, AMX, CRO, STR, OFL, CPX, CHL, GEN, PFX, ERY, NIT, TET	12
	1	AUG, AMX, CRO, STR, CPX, CHL, GEN, COT, ERY, NIT.	10
	1	AUG, AMX, CRO, STR, OFL, CPX, CHL, GEN, PFX, COT, ERY, NIT, TET	13
	1	AUG, AMX, STR, OFL, CHL, PFX, COT, ERY, NIT, TET	10
	1	AUG, AMX, CRO, STR, CHL, COT, ERY.	7
<i>Flavobacterium</i> species	2	AUG, AMX, CRO, STR, CHL, GEN, PFX, COT, ERY, NIT, TET.	11
	2	AUG, AMX, CRO, STR, CPX, CHL, GEN, COT, NIT, TET	10
<i>Proteus</i> species	2	AUG, AMX, CRO, STR, CHL, GEN, COT, NIT.	8
	2	AUG, AMX, CRO, STR, OFL, ERY, TET	7
<i>Bacillus</i> species	1	AUG, AMX, CRO, STR, OFL, CPX, CHL, GEN, PFX, COT, ERY, NIT, TET.	13
	1	AUG, AMX, OFL, CPX, COT, ERY, NIT, TET	8

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Full Length Research Paper

## ***Klebsiella variicola*, a nitrogen fixing activity endophytic bacterium isolated from the gut of *Odontotermes formosanus***

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Termites play an important role in promoting agricultural ecosystem balance and the degradation of lignocelluloses, but also have caused considerable damage to agriculture, forestry and buildings. Previous studies showed that there were a lot of nitrogen-fixing bacteria in the termite gut, and up to 60% of the nitrogen in termite organs came from the nitrogen fixation of symbiotic microorganisms which lived in the hindgut of termites. We obtained a endophytic bacterium from *Odontotermes formosanus*, and the morphology, physiochemical characteristics, 16S rDNA sequence, fatty acid dyeing and SDS-PAGE analysis of strain HUB-IV-005 were very similar to those of *Klebsiella variicola*. Strain HUB-IV-005 had nitrogen fixation activities because it could grow on nitrogen deficient medium under anaerobic condition with nitrogen gas in the headspace. In addition, the nitrogenase gene from HUB-IV-005 was cloned and transformed into *E. coli*, and we identified that the expressed protein was a nitrogenase (iron protein) by mass spectrometry. Strain HUB-IV-005 had nitrogen fixation activities and belonged to the species *K. variicola*. The results provided the basis for studying the nitrogen-fixing mechanism in the termite gut, and nitrogen fixation also provided useful information for further explaining the mechanism of the termite's biological nitrogen fixation.

**Key words:** *Odontotermes formosanus*, *Klebsiella variicola*, endophytic bacteria, nitrogen fixation, protein separation and purification.

### INTRODUCTION

Termites not only attack living trees, but also degrade wood and other celluloses in the terrestrial environment (Long et al., 2010). They can damage many other kinds of materials, from paper, fabrics and wood structures to non-cellulose materials such as asphalt, asbestos, bitumen, lead and metal foils (Lax and Osbrink, 2003; König, 2006; Monica et al., 2009). Biodegradation of wood caused by termites is recognized as one of the most serious problems for wood utilization. The annual damage to trees and wooden structures is estimated as \$300 million dollars in New Orleans, LA, USA, and the

cost for damaging houses, trees, dam, and buried cables is estimated at \$250-300 million dollars in China (Zhong and Liu, 2002; Zhang et al., 2008). The total global damage by termites has been estimated to exceed \$3 billion annually worldwide (Cheng et al., 2007). Therefore, there is a huge potential market for effective termiticides to control the propagation of termites.

On the other hand, termites are important social creatures; they play important roles in the material cycling in nature and carbonization process. They are typical organisms that usually thrive on nitrogen-poor food

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(Liu et al., 2010), such as wood materials rich in cellulose, hemi-cellulose and lignin (Yamin, 1981; Oppert et al., 2010). Previous research has shown that the nitrogen content of termites' organs are similar to that of other animals, nitrogen-fixing process happened in the termite gut, and up to 60% of the nitrogen in termites' organs comes from the nitrogen fixation of symbiotic microorganisms living in the hindgut (Sarcinelli and Schaefer, 2009; Shi et al., 2010). The termite's hindgut has been recognized as the world's smallest bioreactor, and in decades it has attracted the attention of microbial researchers (Mei et al., 2002).

There are a lot of protozoans in the lower area of the termites' guts, symbiotic protozoans play very important roles in the digestion of lignocelluloses and there is some sort of symbiosis between the lower gut area of termites and their symbiotic animal relationships. Studies have shown that the basis of this symbiotic relationship is protozoan degradation of lignocelluloses material. Nitrogen fixation has been demonstrated in all termite families. Given their global distribution, termites may have widespread importance in the biogeochemical cycling of nitrogen in terrestrial ecosystems. Termites may contribute significant amounts of nitrogen to terrestrial ecosystems with their microbial flora of nitrogen-fixing bacteria (Curtis and Waller, 1998). As early as in 1973, Brenak adopted a combination of experimental methods with acetylene reduction and nitrogen fixation indicators; it confirmed that bacteria could fix nitrogen in the lower area of the termite's body (Brenak et al., 1973). In 1996, Ohkuma successfully cloned a *nifH* gene fragment encoding nitrogenase iron protein from the *Reticulitermes speratus* mixed intestinal microbial flora, which further confirmed that nitrogen-fixing microbes exist in the termite's intestine (Ohkuma et al., 1996). The *nifH* gene encodes nitrogenase and its main structure is conservative. The *nifH* nitrogenase activity contributes largely to the termite nitrogenase activity, and there is a high similarity of amino acid sequences derived from other wood-feeders' nitrogenase and termite's nitrogenase *nifH*. It explains why in termites *in vivo* nitrogenase diversity is related to the type of host life and its phylogenetic position (Inward et al., 2007).

We have isolated more than 183 endophytic bacterial strains from *Juniperus virginiana* grown in Louisiana and *Chamaecyparis lawsoniana* grown in Oregon, USA and the intestine of *Odontotermes formosanus* that lived in Logia Hill of Wuhan, China. One strain HUB-IV-005 with strong nitrogenase fixation activity was selected for this study. Through the analysis of morphological, physiological, biochemical characteristics, 16S rDNA sequences, fatty acid dyeing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the expressed protein of nitrogenase (*nifH*) gene from HUB-IV-005 was a nitrogenase (iron protein) by mass spectrometry, we demonstrated that the strain HUB-IV-005 had nitrogen fixation activities and belonged to the species

*Klebsiella variicola*. These results showed the role of nitrogen fixing bacterium in the microbial ecology of termite gut ecosystem, and provided the basis for studying the nitrogen-fixing mechanism in the termite gut, and facilitated the development of nitrogen-fixing microorganism resources.

## MATERIALS AND METHODS

### Strains for bioassay

The strain HUB-IV-005 with nitrogen fixation activity, which was isolated from the gut of *O. formosanus*, was stored in the Key Laboratory of Microbiology, College of Life Science and China center for type culture collection (CCTCC NO: M 20113132), Heilongjiang University. The reference *K. pneumoniae* strain was obtained from China General Microbiological Culture Collection Center. *Escherichia coli* DH5 $\alpha$  and BL21 were kindly provided by the Laboratory of Molecular Biology, Heilongjiang University.

### Morphological examination of the strain HUB-IV-005

Strain HUB-IV-005 was activated at 37°C on a 9 cm plate of beef extract and peptone medium containing 3 g/L beef extract, 10 g/L peptone and 5 g/L NaCl at 37°C for 10-24 h (Shen and Chen, 2007). The bacterium was inoculated at three different spots on the plate and then cultured at 37°C for 12-24 h. As described previously, the strain HUB-IV-005 was detected and identified to the genus and species levels based on the morphological characteristics (John et al., 1994; Shen and Chen, 2007). The morphology of the strain HUB-IV-005 was examined with both light microscope and transmission electron microscope. Somatic diameters were measured after Gram staining, spore staining, negative staining, and silver staining of the flagella. Digital micrographs of colonies were taken with a Coolpix 995 camera (Nikon, Tokyo, Japan).

### Physiological and biochemical characteristics of the strain HUB-IV-005

Physiological and biochemical characteristics of the strain HUB-IV-005 and reference strains were examined as previously described (Ladha et al., 1983; Tan et al., 2009).

### Molecular analysis of the strain HUB-IV-005

Culture and collection of mycelia were carried out as previously described (Liu et al., 2008). The 16S rDNA sequences were amplified using polymerase chain reaction (PCR) with primer pairs 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACGGTTACCTTGTTACGACTT-3'. The PCR reactions were carried out as previously described (Zhao et al., 2011). The obtained sequences were submitted to GenBank for homology search with BLAST (<http://rdp.cme.msu.edu> and <http://ncbi.nlm.nih.gov>). A phylogenetic tree was constructed from the evolutionary distances by PHYLIP (version 3.57c).

### Fatty acid dyeing and SDS-PAGE analysis of the strain HUB-IV-005

Fatty acid dyeing was carried out as previously described (Lu et al., 1997). 40 mg wet bacterium were collected in a clean culture tube, then 1.0 ml of 15% (w/v) NaOH-methanol was added into the culture tube. The tubes were securely sealed with Teflon lined caps,

caps, vortexed briefly and heated in a boiling water bath for 30 min. The cooled tubes were uncapped, and 2.0 ml of 25% (v/v) HCl-methanol was added. The tubes were capped, vortexed briefly, and heated for 10 min at 80°C. One millilitre of hexane-methyl tert butyl ether (1:1, v/v) was added to the cooled tubes, followed by recapping and gentle tumbling the tubes for about 10 min. The tubes were uncapped and the aqueous (lower) phases were pipetted out and discarded. About 3 ml of 12% NaOH was added to the organic phase remaining in the tubes. Then the tubes were recapped, and tumbled for another 5 min. About 2/3 of the organic phase was pipetted into a GC vial, which was capped and ready for analysis. GC operating conditions was in accordance with the method described of Igor et al. (2005).

For SDS-PAGE analysis, 8% acrylamide gel was used and protein extraction of the *K. variicola* isolates was carried out as previously described (Alavandi et al., 2001). Discontinuous buffer system with 10% resolving gels and 5% stack gels was used. All samples were heated for 5 min in boiling water and electrophoresed with constant voltage of 120 V until the dye-front reached the bottom of the gel. The protein bands were visualized by Coomassie Brilliant blue-R250 staining.

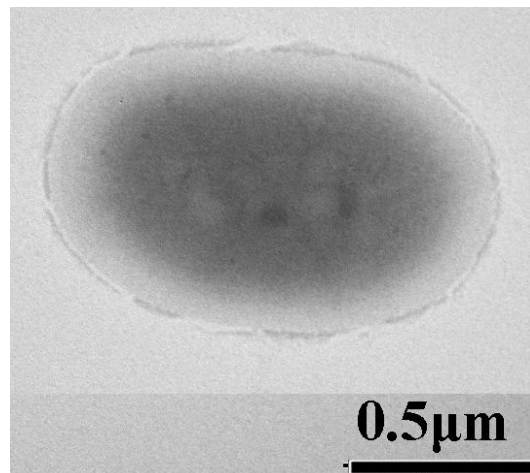
#### Nitrogen fixation assay of the strain HUB-IV-005

The strain HUB-IV-005 was activated, and cultured for different times at 37°C on a plate of nitrogen free medium composed of sucrose 10 g/L, malic acid 5.0 g/L,  $K_2HPO_4 \cdot H_2O$  0.1 g/L,  $KH_2PO_4 \cdot H_2O$  0.4 g/L,  $MgSO_4 \cdot 7H_2O$  0.2 g/L, NaCl 0.1 g/L,  $CaC_12 \cdot 2H_2O$  0.02 g/L,  $FeCl_3$  0.01 g/L,  $Na_2MoO_4 \cdot H_2O$  0.002 g/L and agar 7.5 g/L, pH 7.2 (Zhang et al., 2000).

Bacterial suspension 100  $\mu$ l of strain HUB-IV-005 and 2 ml of liquid medium composed of sucrose 10 g/L, malic acid 5.0 g/L,  $K_2HPO_4 \cdot H_2O$  0.1 g/L,  $KH_2PO_4 \cdot H_2O$  0.4 g/L,  $MgSO_4 \cdot 7H_2O$  0.2 g/L, NaCl 0.1 g/L,  $CaC_12 \cdot 2H_2O$  0.02 g/L,  $FeCl_3$  0.01 g/L,  $Na_2MoO_4 \cdot H_2O$  0.002 g/L and peptone 0.2 g, pH 7.2 (Zhang et al., 2000) was added to culture bottles, and then cultured at 37°C for 24 h. Rubber stoppers were then replaced with cotton stoppers so that 0.5 ml air was exhausted from the sample bottles using 1 ml syringe, and 0.5 ml ethylene was reinjected. The holes produced by syringes were sealed by parafilm; the bottles were cultured at 30°C for another 24 h. Then 5.0  $\mu$ l gas as aforementioned was exhausted from the bottle with 10.0  $\mu$ l trace syringe. The ethylene quantity was tested by GC. Then nitrogen fixation activity was calculated according to ethylene quantity. Enzyme activity was demonstrated by gram-molecule of produced ethylene per hour per milliliter of the broth [nmol ethylene/ (h·ml)]. Three replicates were used for each test. The result was the average of three replicates.

#### Cloning and expression of *nifH* gene from the strain HUB-IV-005

The *nifH* gene sequences were amplified by PCR with primer pairs P3 (5'-GAATTCTCAGGCCGCGTTCCTTCAG-3') and P4 (5'-CATATGATGACCATGCGTCAATGCG-3'). The plasmid of pET28a was extracted using TIANprep Mini Plasmid Kit (DP103-02, TIANGEN). The recombinant plasmids were identified by *EcoRI* and *NdeI* digestion and PCR amplification. Then the *nifH* gene was ligated overnight with pET28a vector by using  $T_4$  DNA ligase. The protein expression was induced by the addition of IPTG, which was inoculated into 20 ml of Luria-Bertani (LB) medium that contained 50  $\mu$ g/ml kanamycin. The affinity purified proteins were analyzed by SDS-PAGE (Wu and Liu, 2012) and stained by Coomassie blue; then it was bleached using a bleaching solution. Gel-spot at the theoretical pI and molecular weight was analyzed using mass spectrometry (Li et al., 2004). Tryptic digest was analyzed on a nanoESI-MS/MS (QSTAR XL hybrid quadrupole (Q) TOF). The pep-



**Figure 1.** Morphology and capsular of the endophytic bacteria HUB-IV-005 under a transmission electron microscope (magnification, 50000 $\times$ ).

tides were separated on a PepMap100  $C_{18}$  column using acetonitrile and water. The MS/MS data were submitted to Mascot software for searching the MS/MS spectra against a local version of NCBI nr.

#### Statistical analysis

The Scheffe multiple comparison procedure from the SAS statistical program was employed to evaluate differences in nitrogenase activity test. Results with  $p < 0.05$  were considered statistically significant. All the experiments were repeated three times and each measured in triplicate. All results were expressed as mean  $\pm$  standard deviation (SD).

## RESULTS

#### Morphological characterization

The colony of strain HUB-IV-005 was white without gloss, translucent, round, regular edges, which was moist on the surface and not easy to pick up with loops in the nitrogen free medium. Strain HUB-IV-005 was Gram-negative, and the cells were short rod-shaped, arranged single or short chain-like arrangements. The cell size of the strain were in accordance with the reference strain (the cell size was 0.2-1.2  $\times$  0.4-5.0  $\mu$ m), with a cell size of 0.5-0.8  $\times$  1.0-2.0  $\mu$ m, and there were no spores or movable flagella around the cell but thicker capsular (Figure 1).

#### Physiological and biochemical characteristics of strain HUB- IV-005

The basic biochemical tests confirmed the isolate as *K. variicola*. As shown in Table 1, the physiological and biochemical characteristics of strain HUB-IV-005 were the same as those of the reference *K. variicola* strain. Strain HUB-IV-005 was Gram-negative, citrate utilization positive,

**Table 1.** Physiological and biochemical characteristics of strain HUB-IV-005.

Characteristic	Strain HUB-IV-005	Reference strain
Gram staining	-	-
Citrate utilization	+	+
Spore staining	-	-
Capsule stain	+	+
Semisolid puncture	-	-
Starch hydrolysis	+	+
Fat hydrolysis	-	-
Gelatin hydrolysis	-	-
Urea utilization	+	+
H <sub>2</sub> S	-	-
Indole production	-	-
V-P reaction	+	+
Methyl red	-	-
Urinary enzyme	+	+
Sucrose fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
Maltose fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
D-sorbitol fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
Rhamnose fermentation test	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
Mannitol fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
Lactose fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>
Trehalose fermentation	A <sup>+</sup> B <sup>+</sup>	A <sup>+</sup> B <sup>+</sup>

+indicates positive reaction;indicates negative reaction; A<sup>+</sup> indicates acid production; B<sup>+</sup> indicates gas production.

spore staining negative, capsule staining positive, semi-solid puncture negative, starch hydrolysis positive, fat hydrolysis negative, gelatin hydrolysis negative, H<sub>2</sub>S test positive, urea utilization positive, indole production negative, V-P reaction positive, methyl red positive and urinary enzyme positive. The isolate fermented glucose, lactose, sucrose, maltose, acid and gas.

### Molecular analysis

The 16S rDNA of strain HUB-IV-005 was successfully amplified using PCR with an expected size of 1,579 bp. After sequencing, this newly identified sequence have been submitted and deposited into GenBank (Accession number GQ892930). After homology searching against GenBank or the proprietary bacteria DNA database, the sequence of strain HUB-IV-005 were found to share 99% similarity with those of *K. variicola*. A phylogenetic relationship was established through the alignment and analysis of homologous nucleotide sequences among these bacteria species (Figure 2). We found that strain HUB-IV-005 was the closest to the genus *Klebsiella*.

### Fatty acid dyeing and SDS-PAGE analysis

Fatty acid dyeing analysis showed that a peak was phenol, 2, 4, 6-tris (1-methylethyl) - with a chemical formula

of C<sub>15</sub>H<sub>24</sub>O, b peak was dibutyl phthalate with a chemical formula of C<sub>16</sub>H<sub>22</sub>O<sub>4</sub>, c peak was 1-decanol, 2-hexyl- with a chemical formula of C<sub>16</sub>H<sub>34</sub>O, and d peak was hexacosyl acetate with a chemical formula of C<sub>28</sub>H<sub>56</sub>O<sub>2</sub> (Figure 3). SDS-PAGE analysis showed that the protein banding of strain HUB-IV-005 was consistent with those of the reference *K. variicola* strain (Figure 4).

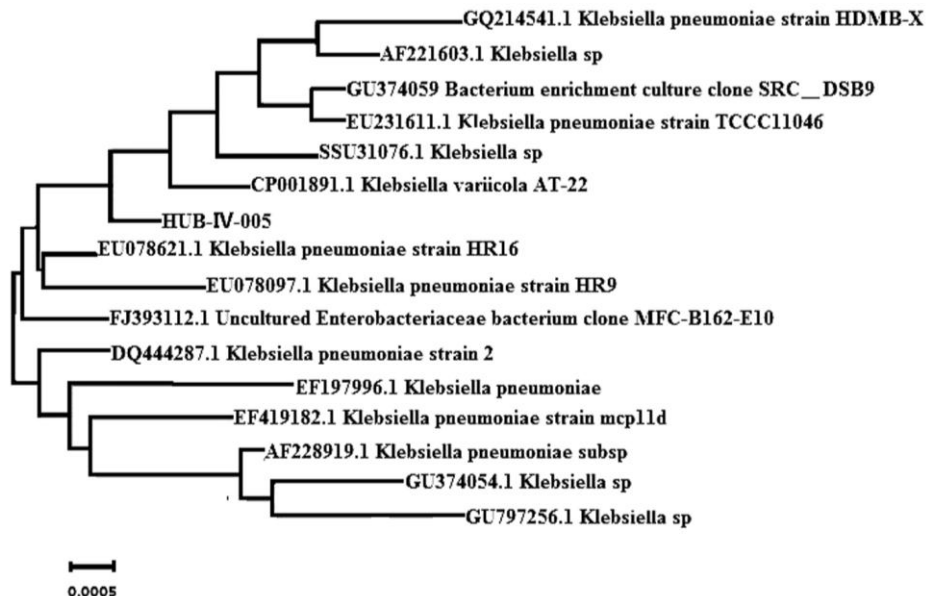
According to phylogenetic analysis, fatty acid dyeing and SDS-PAGE analysis, strain HUB-IV-005 was classified to the genus *Klebsiella* as a species of *K. variicola*.

### Nitrogenase activity detection

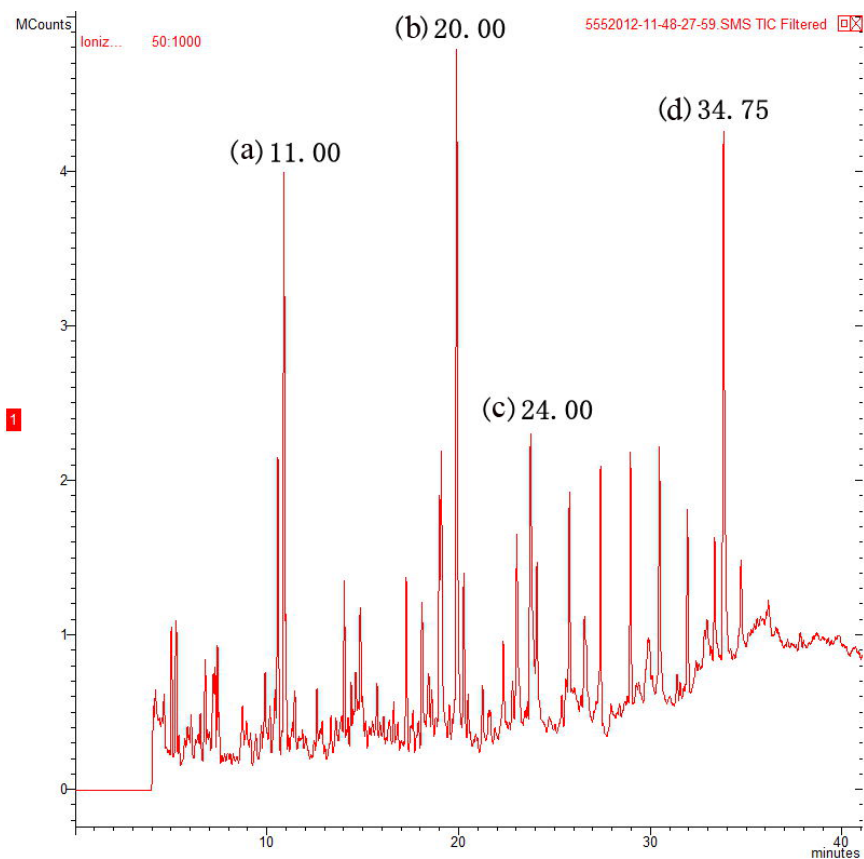
The strain HUB-IV-005 was able to grow on the nitrogen free medium. The colony of the strain was white and translucent with round and regular edges, which were moist on the surface and not easy to pick up with loops. The strain HUB-IV-005, an azotobacter in the gut of termite, showed a high level of nitrogenase activity of 167.46±4.40 nmol C<sub>2</sub>H<sub>4</sub>/ (h·ml).

### Cloning and sequence analysis of nifH gene

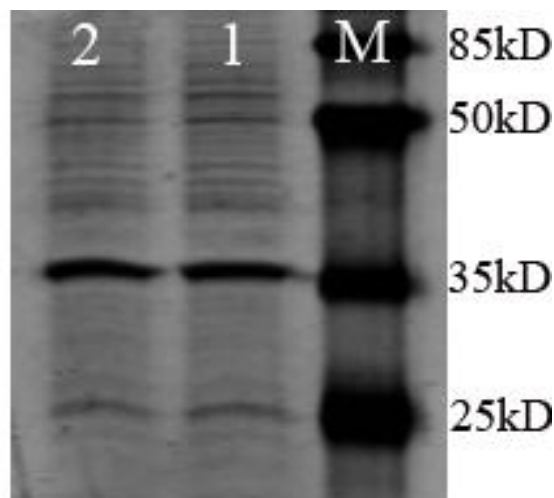
The *nifH* gene of the strain HUB-IV-005 was successfully amplified by PCR with an expected size of 882 bp (Figure 5). To confirm the PCR product was *nifH* gene, the PCR product was inserted into pMD18-T vector and transformed



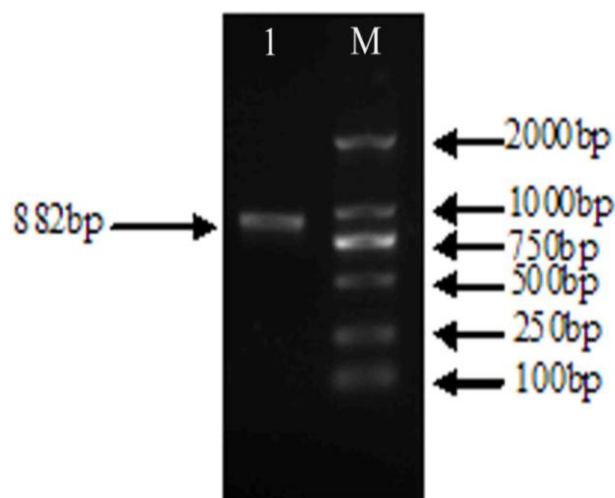
**Figure 2.** Phylogenetic tree showing the relationship of the strain HUB-IV-005 with other related bacterial species from GenBank based on their homologous sequences of 16S rDNA.



**Figure 3.** GC-MS spectrum of the extract from the strain HUB-IV-005 by fatty acid dyeing analysis. a peak was phenol, 2, 4, 6-tris (1-methylethyl) - with a chemical formula of  $C_{15}H_{24}O$ ; b peak was dibutyl phthalate with a chemical formula of  $C_{16}H_{22}O_4$ ; c peak was 1-decanol, 2-hexyl- with a chemical formula of  $C_{16}H_{34}O$ ; d peak was Hexacosyl acetate with a chemical formula of  $C_{28}H_{56}O_2$ .

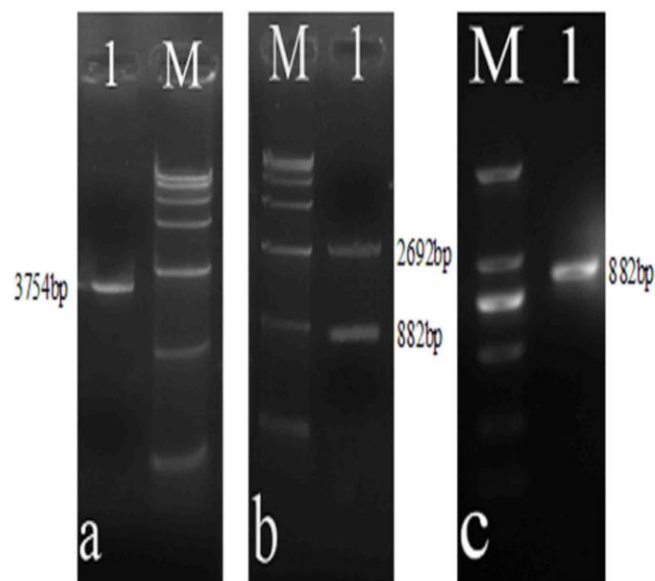


**Figure 4.** Protein banding analysis of proteins extracted from the strain HUB-IV-005 using SDS-PAGE. Lane 1: protein banding from the strain HUB-IV-005; Lane 2: protein banding from the reference *K. variicola* strain; M: protein marker including 80 kDa, 50 kDa, 35 kDa and 25 kDa.



**Figure 5.** Analysis of PCR product by agarose gel electrophoresis. Lane M: DNA marker DL2000; Lane 1: PCR product of the *nifH* gene.

into *E. coli* DH5 $\alpha$ . The recombinant plasmid pMD18-T-*nifH* were extracted from the positive clones and analyzed by electrophoresis (Figure 6a). The recombinant plasmid pMD18-T-*nifH* was identified by *EcoRI* and *NdeI* enzyme digestion (Figure 6b) and PCR amplification (Figure 6c), and the positive clones were sequenced, and analyzed by BLAST. It was 882 bp in length and 99% homology with the *nifH* gene sequences in GenBank. This sequence was submitted to GenBank, and the accession number was JX 570695.

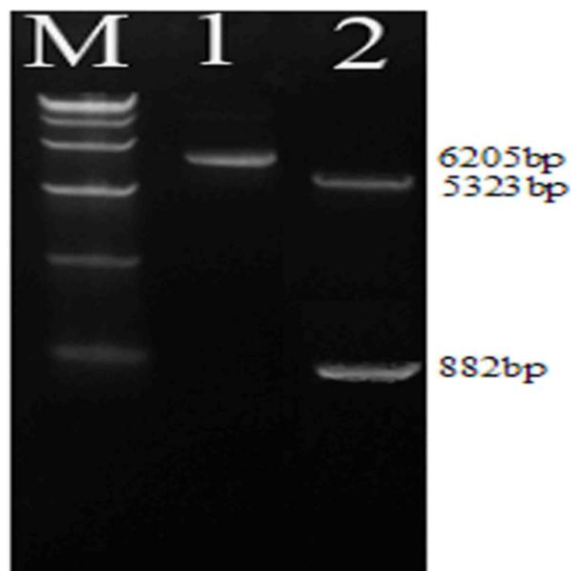


**Figure 6.** The extraction and identification of recombinant plasmid pMD18-T-*nifH*. a: the extraction of recombinant plasmid pMD18-T-*nifH* (Lane M: DNA marker DL15000; Lane 1: the extracted recombinant plasmid pMD18-T-*nifH*); b: identification of the extracted recombinant plasmid pMD18-T-*nifH* by *EcoRI* and *NdeI* enzyme digestion (Lane M: DNA marker DL15000; Lane 1: enzyme digestion of the plasmids); c: identification of the extracted recombinant plasmid pMD18-T-*nifH* by PCR (Lane M: DNA marker DL15000; Lane 1: PCR amplification of the plasmids).

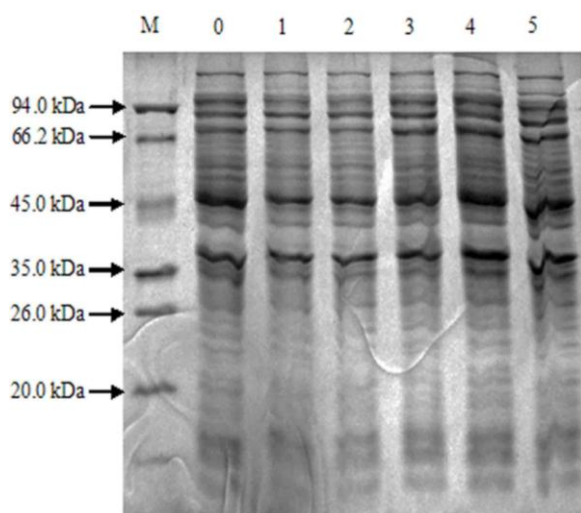
#### Analysis and expression protein identification of the *nifH* gene expression

The plasmid pET28a was extracted and analyzed by electrophoresis (Figure 7), and then the *nifH* gene was ligated with pET28a and identified by *EcoRI* and *NdeI* enzyme digestion (Figure 7), and then the recombinant plasmid pET28a-*nifH* was transformed into the *E. coli* BL21. The SDS-PAGE analysis showed that the expression products of the *nifH* gene could be efficiently recovered to a high degree of purity by single-step affinity chromatography. The protein was furthermore found to be recovered as predominantly full-length products of expected size. The protein of *nifH* gene in *E. coli* BL21 containing pET28a-*nifH* was expressed after IPTG induction at 4 and 5 h, while there was no specific proteins in the control containing the plasmid pET28a (Figures 8 and 9).

After mass spectrometric identification of the target protein, two credible identification results were gained (Table 2). It was *K. variicola* nitrogenase iron protein, and its molecular weight was 32.463 kDa, an isoelectric point of 4.72, a score of 731, a total of 39 peptides, and coverage of 46%. The other one was *Delftia tsuruhatensis* nitrogenase iron protein. The molecular weight was 31.690 kDa, an isoelectric point of 4.68, a score of 224, and a total of 14 peptides and 21% coverage. Therefore,



**Figure 7.** The extraction and identification of recombinant plasmid pET28a-*nifH*. Lane M: DNA marker DL15000; Lane 1: the extracted recombinant plasmid pET28a-*nifH*; Lane 2: identification of the extracted recombinant plasmid pET28a-*nifH* by *EcoRI* and *NdeI* enzyme digestion.

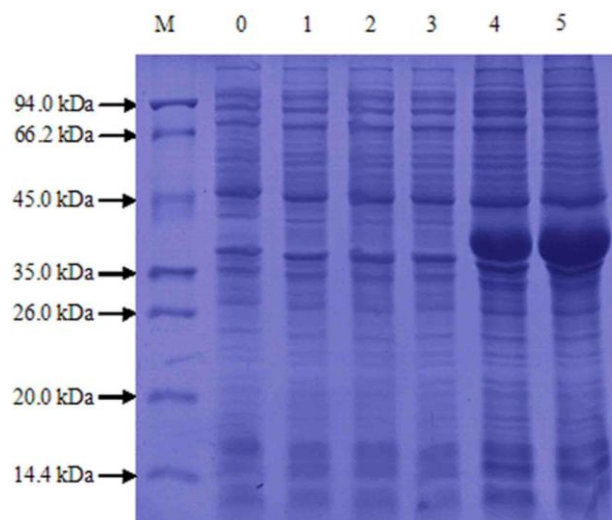


**Figure 8.** SDS-PAGE analysis of pET28a (without the *nifH* gene) transformed into the *E. coli* BL21. Lane M: protein molecular weight marker; Lane 1-6: IPTG induction for 0, 1, 2, 3, 4, 5 h, respectively.

based on the mass spectrometry results, *nifH* genes could be properly expressed as the nitrogenase iron protein in *E. coli* BL21 (Hou et al., 2007).

## DISCUSSION

Termite gut has become one object of study in microbial diversity on the earth, because there are more than a



**Figure 9.** SDS-PAGE analysis of expressed proteins from the recombinant plasmid pET-28a-*nifH* in the *E. coli* BL21. Lane M: protein molecular weight marker; Lane 1-6: IPTG induction for 0, 1, 2, 3, 4, 5 h, respectively.

thousand species of termites, their feeding habits and structures of the intestine are different. Nitrogen limits plant growth in many ecosystems (Fiore et al., 2010). Previous studies showed that there are a lot of nitrogen-fixing bacteria in the termite gut, and up to 60% of the nitrogen in termite organs came from the nitrogen fixation of symbiotic microorganisms which lived in the hindgut of termites. It was been confirmed that the nitrogen fixing bacteria in the hindgut of the termites are important because the termite's diet is nitrogen deficient. Termite's gut symbiotic microbial diversity is extremely rich. The digestion of cellulose and hemicellulose by low termites mainly relies on hindgut symbiotic microorganisms (including protozoan). The high termites' digestion of lignin relies on digestive juices secreted by the salivary glands and midgut. The capability of nitrogen fixation may meet the termites' metabolic needs. The level of nitrogen fixation depends on the species and composition of symbiotic microorganism in the gut of termites. We have isolated endophytic bacteria with nitrogen fixation activity from the gut of *O. formosanus*; one of them is the strain HUB-IV-005. In addition, the morphological and physiological, biochemical characteristics, 16S rDNA sequences, fatty acid dyeing and SDS-PAGE analysis showed a close relationship of strain HUB-IV-005 with the genus *Klebsiella*. Molecular identification has been increasingly used as a powerful tool supplementary to the traditional systematic classification. In our study, the *nifH* gene from HUB-IV-005 was successfully cloned, and the expressed protein of the *nifH* gene was a nitrogenase (iron protein), indicating that the strain HUB-IV-005 represents a nitrogen fixation bacterium from *O. formosanus*. Based on these results, we named strain HUB-IV-005 as *K. variicola*.

According to previous studies, nitrogen-fixing bacteria

**Table 2.** Protein identification from MS/MS.

Accession no.	Protein name	Organism	pI/MW exp (kDa)	MW Theo (kDa)	Mascot score	Sequence coverage (%)	Pep
gjl206580625	Nitrogenase iron protein	<i>Klebsiella pneumoniae</i> 342	4.72/ 32.463	32	731	46	39
gjl45269096	<i>nifH</i>	<i>Delftia tsuruhatensis</i>	4.68/ 31.690	32	224	21	14

is capable of nitrogen fixation or utilization. To be specific, nitrogen-fixing bacteria can provide vitamins for improvement of activities of other microbial life, however cellulose decomposing bacteria decompose cellulose to provide a carbon source for nitrogen-fixing bacteria. Accordingly, azotobacter and cellulose decomposing bacteria can work mutually (Hethene et al., 1992). It is difficult to complete the biological degradation of cellulose alone; but it can work well with two or more microorganisms. Cheng et al. (2007) made azotobacter and cellulose-decomposing bacteria a mixed culture. In addition, the number of bacteria increased when the cellulose-decomposing bacteria and nitrogen-fixing bacteria were cultured together (Zhou et al., 2007). To further characterize the extracted product from strain HUB-IV-005, the characterization of nitrogen fixation is necessary. In our study, we investigated the nitrogenase activity of strain HUB-IV-005, that was able to grow on the nitrogen free medium. At the same time, we also detected nitrogenase activity of strain HUB-IV-005 by acetylene reduction method. Strain HUB-IV-005, an azotobacter in the gut of termite, showed a high level of nitrogenase activity of  $167.46 \pm 4.40$  nmol  $C_2H_4$  (h·ml) $^{-1}$ . Under anaerobic or microaerobic conditions, *K. variicola* was considered as an associative nitrogen fixer (Ladha et al., 1983). Isolates of *Klebsiella* have been found in living or decaying wood, bark and composted wood (Descamps et al., 1983). Therefore, it is reasonable to consider that the *K. variicola* may play an important role in the hindgut microbial ecology. The presence of a nitrogen fixing spirochete has also been identified in the termite gut (Lilburn et al., 2001).

Although researchers have shown that the termite gut has a large number of nitrogen-fixing bacteria, pure cultures of nitrogen-fixing bacteria were rarely reported. In the nitrogenase discovered to date, one was the *nifH* gene encoding the molybdenum and ferritin protein. It has been recognized as the best nitrogen-fixing capacity, the complete set of structure and function in nitrogenase system. *nifH* gene cluster was found in *Klebsiella* nitrogen-fixing bacteria. Nitrogenases were expressed by a relatively conservative *nifH* gene and the expression levels of *nifH* gene contribute largely to the organism nitrogenase activity. For this reason, it was appropriate to study the bacterial molecular diversity. However, only a small amount of the *nifH* gene could be preferentially transcribed and expressed, most of the *nifH* gene could not be successfully expressed. The *nifH* gene in our study was cloned and the *nifH* gene was expressed in order to understand the nitrogen-fixing mechanism in

termites; at the same time, we separated and purified the nitrogenase, which was produced by the *nifH* gene, and the sequence alignment with *K. variicola* was 99% sequence identity. Our results provided the basis for studying a nitrogen-fixing mechanism in the termite gut, and facilitated the development of nitrogen-fixing micro-organism resources and developing new termite control methods with important theoretical and practical potential.

## ACKNOWLEDGEMENTS

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## Full Length Research Paper

## Pathogenicity of *Staphylococcus sciuri* in murine *in vivo* model

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This study was conducted to assess the pathogenic potential of exfoliative toxin gene-positive *Staphylococcus sciuri*. Twenty eight eight weeks-old mice were used for the study. The animals were randomly assigned into four groups of 7 mice per group. Groups 1 and 2 were infected with  $1 \times 10^8$  colony-forming unit (cfu)/ml of the organism intraperitoneally (i.p) and subcutaneously (s.c) respectively, while groups 3 and 4 were inoculated with sterile phosphate buffered saline (PBS) i.p and s.c, respectively. Groups 3 and 4 served as the controls. The mice were monitored daily for 15 days for skin lesions, morbidity and mortality, and rectal temperatures. The body weights of the individual mouse were also taken at two days interval. The mean values of rectal temperatures and body weights were subjected to one-way analysis of variance (ANOVA) and a value of  $p < 0.05$  was considered significant. The morbidity rates for groups 1 and 2 were 57 and 71%, respectively, while mortality rate for both groups was 11%. No mortality was recorded in groups 3 and 4. Skin lesions were recorded in all the mice in *S. sciuri*-infected groups, while no skin lesions were recorded in the control groups. Significant ( $p < 0.05$ ) increase in rectal temperatures were recorded in the infected groups compared to the control groups. The body weight significantly ( $p < 0.05$ ) reduced in infected groups against the controls. This study has shown that exfoliative toxin gene-containing *S. sciuri* is potentially pathogenic.

**Key words:** *Staphylococcus sciuri*, skin lesion, mortality.

### INTRODUCTION

Coagulase-negative staphylococci (CoNS), with the exception of *Staphylococcus saprophyticus*, are generally considered to be bacteria of doubtful pathogenic potential in humans and animals (Stepanovic et al., 2003). They have long been regarded as harmless skin commensals and dismissed as culture contaminants (Shittu et al., 2004). But with the substantial rise in the incidence of

CoNS reported as causative agents of nosocomial infections following the use of prosthetic valves and other invasive technologies (Peters, 1988; Huebner and Goldmann, 1999; Petinaki et al., 2001; Shittu et al., 2004), a lot of interests in the pathogenic potential of these microorganisms have been generated.

Amongst the CoNS, *Staphylococcus sciuri* (*S. sciuri*) is

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widely distributed in nature and found principally as skin commensal in animal species (Kloos, 1980; Devriese et al., 1985; Stepanovic et al., 2001a; Nagase et al., 2002), but they may colonize humans, and their isolation from various human clinical specimens has been reported (Kloos, 1980; Kloos et al., 1997; Masou et al., 1999; Couto et al., 2000; Nagase et al., 2002). However, from the clinical standpoint, most *S. sciuri* isolates recovered from humans have not been considered important (Shittu et al., 2004) and at present, only seven cases of *S. sciuri* infection have been established in humans. These include serious infections such as endocarditis (Hedin and Winderstrom, 1998), peritonitis (Wallet et al., 2000), septic shock (Horii et al., 2001), urinary tract infections (Stepanovic et al., 2003), wound infections (Stepanovic et al., 2002; Shittu et al., 2004, Coimbra et al., 2011), pelvic inflammatory disease (Stepanovic et al., 2005) and bacteraemia (Ahoyo et al., 2013).

In animals, *S. sciuri* has been reported to be an invasive pathogen where it caused wound infections (Adegoke, 1986; Devriese, 1990), mastitis in goat (Poutrel, 1984) and cow (Rahman et al., 2005), and a highly fatal exudative epidermitis in piglets (Chen et al., 2007). The isolation of *S. sciuri* from these clinical specimens has generated a lot of interest in the potential of this microorganism to cause infections in humans and animals.

The ability of *S. sciuri* to cause these wide array of diseases, has been attributed to its capacity to produce virulent factors such as deoxyribonuclease (DNase), biofilm, clumping factor, proteinase, lipase, delta toxin, capacity to stimulate nitric oxide production (Stepanovic et al., 2001b) and ability to harbour exfoliative toxin gene (Chen et al., 2007).

Although there are reports on infections caused by *S. sciuri* and possible virulent factors involved, its pathogenic potentials have not been fully investigated. Moreover, its effects on body organs have not been assessed and the exact contribution of its virulent factors in the course of infection is still controversial. The aim of the present study was to investigate the effects of exfoliative gene-positive *S. sciuri* on the skin and some internal organs of mice.

## MATERIALS AND METHODS

The experimental protocol used in this study was approved by the Ethics Committee of the University of Nigeria, Nsukka, and conforms with the guide to the care and use of animals in research and teaching of University of Nigeria Nsukka, Enugu State, Nigeria.

### Animals

Eight-week-old mice of both sexes, weighing between 19 and 22 g were obtained from the laboratory animal unit, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were fed on commercial growers mash (Guinea feeds®) and water was provided *ad libitum*. These mice were acclimatized for 2 weeks in the animal

house at the Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka.

### Pathogen and preparation of inoculum

A strain of *S. sciuri* isolated from the skin of an apparently healthy dog in Nsukka, Nigeria and fully identified and characterized at the Molecular Biology and Biochemistry laboratory, University of Logrono, Spain (Courtesy of Prof. Carmen Torres) was used in the study. The *S. sciuri* strain used is positive for exfoliative toxin A (*eta*) gene. The isolate was maintained on nutrient agar slant at 4°C in the Microbiology unit of the Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. Prior to use, the strain was sub-cultured on mannitol salt agar, incubated aerobically at 37°C for 24 h. Colonies were homogenized in sterile PBS and the turbidity adjusted to correspond to 0.5 McFarland's turbidity standard (equivalent to  $1 \times 10^8$  cfu/ml).

### Animal groups and infection

Twenty-eight adult eight weeks-old mice were randomly assigned into four groups of seven mice per group. Groups 1 and 2 were infected with  $1 \times 10^8$  cfu/ml exfoliative toxin-positive *S. sciuri* intraperitoneally (i.p.) and subcutaneously (s.c.) respectively, while groups 3 and 4 were similarly inoculated with sterile PBS and served as the controls.

### General observation, sample collection and bacterial culture

The animals were observed daily for fifteen days post-infection (dpi) and their physical condition, rectal temperature, morbidity and mortality were recorded. The weights of the individual animals were taken at two days interval. Skin lesions and internal organs (spleen, kidney and liver) of mice that developed skin lesions and/or died were processed for bacteria isolation and histopathology.

Positive culture was confirmed if the morphology and biochemical characteristics of the isolated strain were identical with that of the inoculum. No colony growth in 48 hours was regarded as a negative culture. Systemic infection was defined by positive culture of *S. sciuri* from one or more internal organs.

### Histopathology

Formalin-fixed and paraffin wax-embedded sections of skin lesions, liver, spleen and kidney were stained with haematoxylin and eosin (H and E).

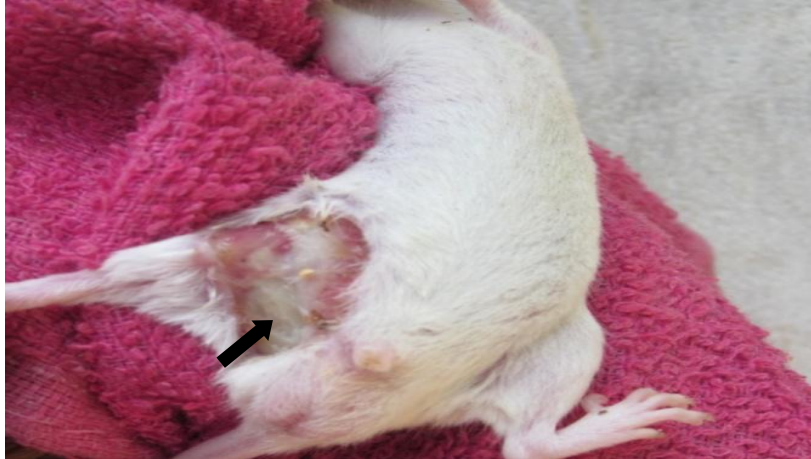
### Statistical analysis

The data collected for morbidity and mortality were subjected to descriptive statistics and expressed in percentages, while the mean values of temperature and body weight parameters were subjected to one-way analysis of variance (ANOVA). Statistical analysis was performed at 5% probability level.

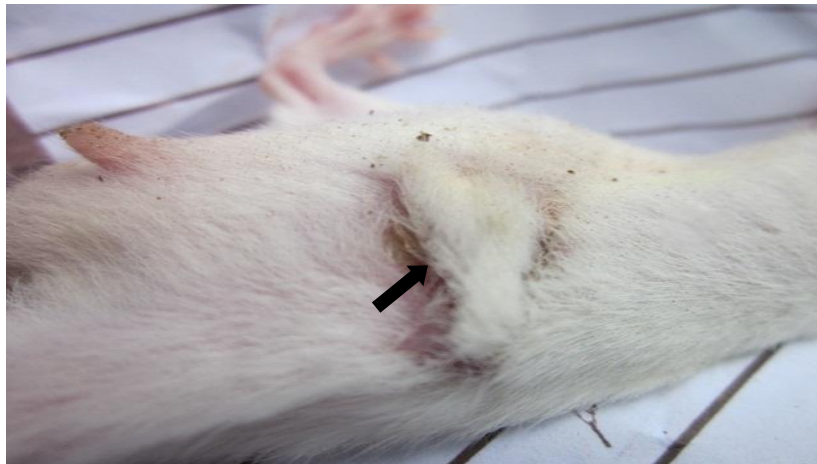
## RESULTS

### Effect of *S. sciuri* infection on clinical signs and skin lesions

Mice inoculated i.p became lethargic by 2 dpi and they



**Figure 1.** Sloughed skin in mouse infected intraperitoneally (i.p) with *S. sciuri* at 8 dpi (arrow).



**Figure 2.** Sloughed skin in mouse infected subcutaneously (s.c) with *S. sciuri* at 7 dpi (arrow).

also had ruffled hair coat, reduced feed and water intake. Some of the mice developed swollen limbs. These signs lasted till end of the experiment by which the morbidity was 57%. In s.c inoculated mice, similar clinical signs were observed, however morbidity by 15 dpi was 71%. These signs were not observed in any of the mice in the control groups.

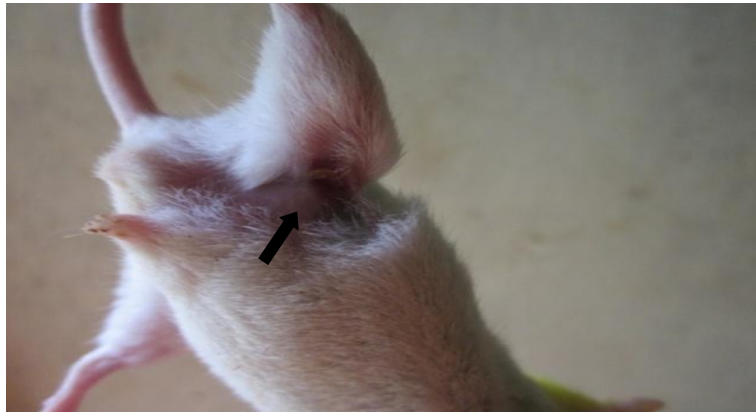
Skin lesions which started as erosions and then progressed to ulcerations developed in all the mice in the *S. sciuri*-infected groups by 6 to 8 dpi (Figures 1 and 2). Healing of the skin lesions started by 9 dpi in both groups and by 15 dpi, healing was complete (Figure 3). No skin lesions were observed in mice in the control groups.

Death of 3 mice occurred by 5 and 6 dpi in groups 1 and 2 respectively, which gave 11% mortality rate for both, while no mortality was recorded in the control groups. Positive bacterial cultures were obtained from

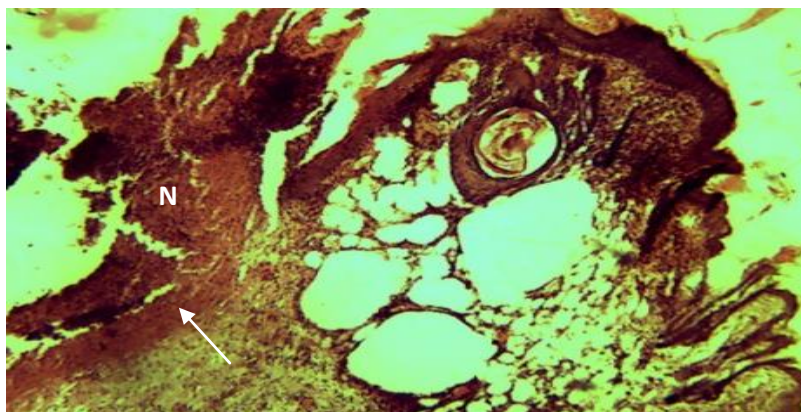
skin lesions and internal organs of all the mice in *S. sciuri*-inoculated groups, while negative cultures were observed in the control groups.

Grossly, the liver of all the mice in the infected groups appeared pale and contained multiple abscesses, visible on both the parietal and visceral surfaces. Microscopically, the important changes observed in the skin of all the mice in the *S. sciuri*-infected groups involved mainly the dermis, characterized by the appearance of dermal abscesses which appeared as necrotic areas admixed with numerous dead and viable polymorphonuclear leucocytes, cellular debris, bounded peripherally by granulation tissue (Figure 4).

Microscopically, their livers showed a mild to moderate periportal leucocytic infiltration comprising of predominantly polymorphonuclear leucocytes, macrophages and a few lymphocytes (Figure 5). Occasionally, multifocal



**Figure 3.** Healed skin in mouse inoculated i.p with *S. sciuri* by 14 dpi (arrow).



**Figure 4.** Skin showing necrotic area (N) admixed with polymorphonuclear leucocytes and cellular debris, bounded peripherally by granulation tissue (arrow).

microabscesses were found, which appeared as multiple aggregates of neutrophils (Figure 6).

#### Effect on body weight

Changes in the mean body weight of *S. sciuri*-infected and uninfected mice are presented in Figure 7. Significant ( $p < 0.05$ ) variations were observed in the mean body weights of mice in the four groups. The mean body weights of mice in the infected groups were lower than those of the uninfected groups.

#### Effect on rectal temperature

Figure 8 shows the effects of *S. sciuri* on the rectal temperature. The mean rectal temperatures of mice in the infected groups did not vary significantly ( $p > 0.05$ ) from each other (groups 1 and 2). They varied signifi-

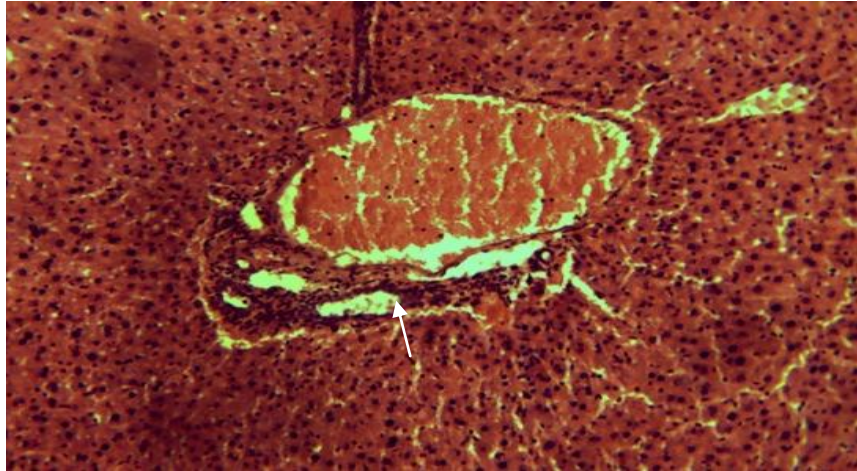
cantly from those of the uninfected groups (group 3 and 4).

#### DISCUSSION

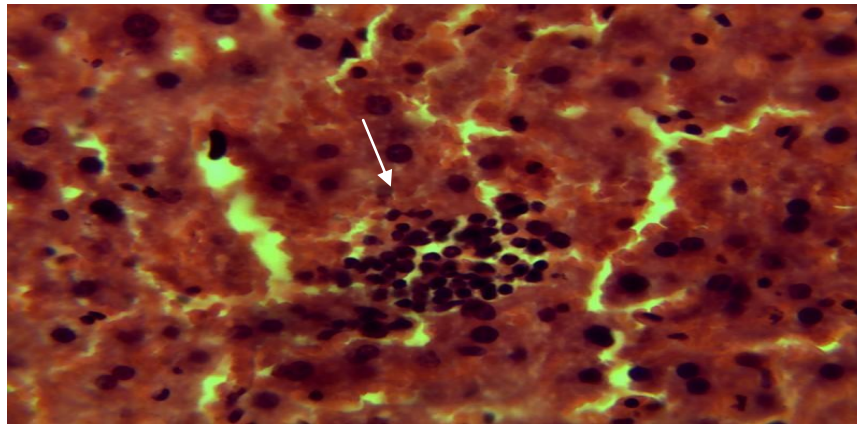
The morbidity rates of 57 and 71% recorded for the intraperitoneally and subcutaneously-infected mice respectively, suggest that the *S. sciuri* isolate used has some pathogenic potential. The pathogenic capability of the inoculum is further supported by the mortalities (11%) as well as the skin lesions recorded in the infected groups.

The mortalities by 5 to 6 dpi which coincided with development of skin lesions by 6 dpi in the infected groups may have resulted due to septicaemia and/or toxæmia. *S. sciuri* isolates have been reported to be invasive pathogens capable of producing variety of toxins (Stepanovic et al., 2001b).

The *S. sciuri* strain used in this study is positive for



**Figure 5.** Section of the liver of i.p inoculated mouse showing a mild periportal leukocytic infiltration (arrow) Mag x100 (H and E).



**Figure 6.** Section of the liver of s. c inoculated mouse showing an aggregation of leukocytes within the liver parenchyma (arrow) Mag x400 (H and E).

exfoliative toxin A gene (*eta*), which encodes exfoliative toxin A production (Sheehan et al., 1992). Exfoliative toxin A has been reported as one of the virulent factors associated with skin lesions produced by coagulase-positive staphylococci such as *S. hyicus*, *S. pseudintermedius* (*intermedius*) and *S. aureus* in pigs, dogs and man respectively (Quinn and Markey, 2003; Bukowski et al., 2010). Therefore, sloughing of the skin of animals in the infected groups observed at 8 dpi may be due to production of exfoliative toxin by the *S. sciuri* isolate inoculated into the animals. This toxin has also been reported to be a possible virulent factor in *S. sciuri* infection (Chen et al., 2007).

Progression of the skin lesions from erosions to ulceration may be due to the ability of the inoculated *S. sciuri* strain to stimulate nitric oxide (NO) production. This capacity has been reported as a possible mechanism of pathogenicity in the course of *S. sciuri* infection

(Stepanovic et al., 2001b). Nevertheless, healing of the skin lesions without treatment by 10 dpi may suggest a kind of self-limiting infection. It may also be that the animals had tremendous ability for skin regeneration. However, in natural infections, secondary infection of the skin lesions by pyogenic bacteria such as *S. aureus* and *Pseudomonas aeruginosa* may delay their healing (Quinn and Markey, 2003).

Significant ( $p < 0.05$ ) variations in the mean body temperatures (with temperature of infected groups higher than that of control groups at each observation period) of mice between 1 and 13 dpi suggests that the *S. sciuri* was able to invade the animals resulting to bacteraemia and fever. The increased body temperature may also be due to production of toxins by the *S. sciuri* strain. But the lack of statistical difference observed by 14 and 15 dpi coincided with the healing period; this suggests that bacteraemia may have receded by that time. Although

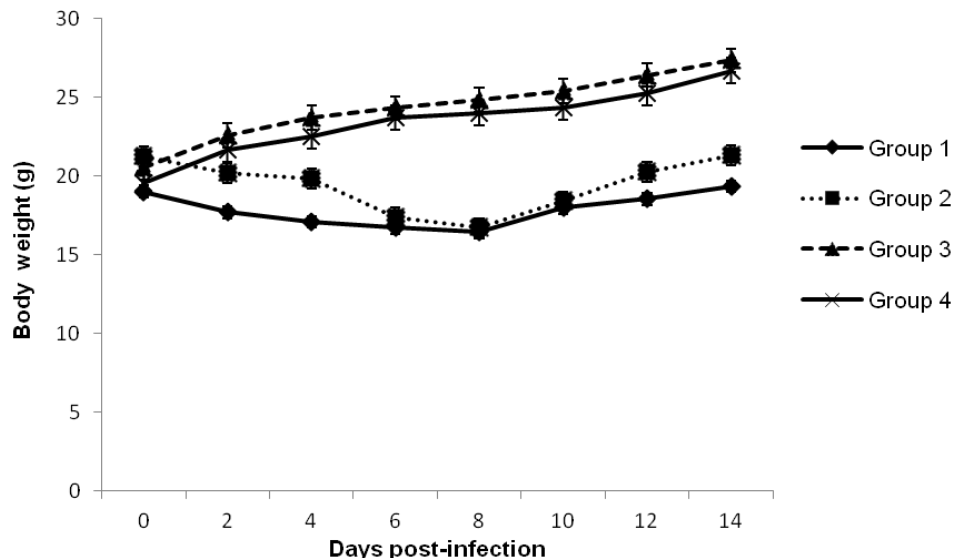


Figure 7. Effect of *S. sciuri* on body weight.

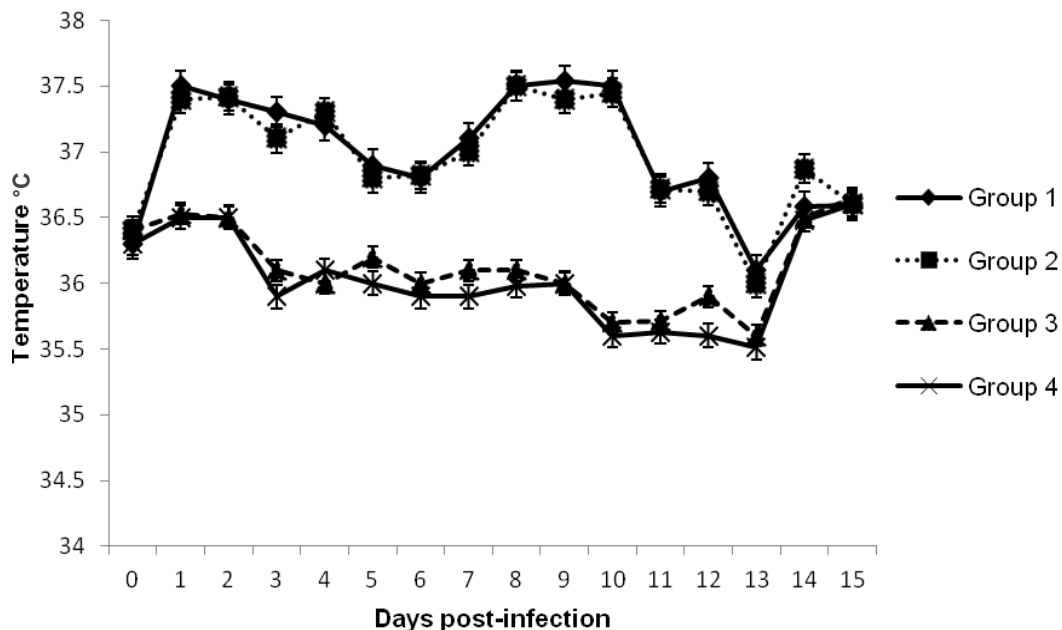


Figure 8. Effects of *S. sciuri* on rectal temperatures.

there was increased body temperature of the *S. sciuri* infected mice, this increase did not exceed the normal body temperature (35 to 38°C) of mice.

The significant ( $p < 0.05$ ) decrease in body weight of the infected groups by 2 to 14 dpi, may be as a result of their reduced feed intake observed in the course of the experiment. Significantly ( $p < 0.05$ ) decreased body weight observed in group 1 compared to 2 at 2 to 4 dpi, may suggest that intraperitoneal inoculation resulted to faster establishment of infection than the subcutaneous route.

Isolation of pure cultures of *S. sciuri* from skin lesions and internal organs and the histopathological findings, further suggests that systemic infection occurred in *S. sciuri*-infected groups. The histopathological changes in form of dermal abscess in the skin and microabscess in liver, may suggest that the organism invaded the organs and caused necrosis which triggered formation of the abscesses. Dermal abscesses are not uncommon findings in *Staphylococcus aureus* infection, and exfoliative toxin C (ExhC) of *S. sciuri* has been suggested

to be a mammalian necrosis inducer (Li et al., 2011). This might be the first report of dermal abscess associated with *S. sciuri* containing exfoliative toxin A gene.

In conclusion, this study has shown that exfoliative gene-positive *S. sciuri* is potentially pathogenic. The pathogenic effect as evidenced by sloughing of the skin in infected mice is probably due to elaboration of exfoliative toxin. There were significant alterations in the temperature, body weight and liver histology of mice infected with the organism. These findings indicate that the significance of *S. sciuri* as a potential pathogen may have been somewhat underestimated. It is therefore suggested that further studies using larger experimental animal models be carried out.

### Conflict of interests

The author(s) have not declared any conflict of interests.

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Review

## Enhancing sustainable development through indigenous fermented food products in Nigeria

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**Indigenous fermented food products are mainly produced by the activities of microorganisms and their preparation remains today as a household art. Fermented food products play very important roles especially in the socio-economic aspect of people in developing countries. In Nigeria, there are large variety of fermented foods and beverages with traditional and cultural value, these fermented food products includes 'Ogi', 'Gari', 'Fufu', 'burukutu', 'Pito', 'Iru', 'Ogiri' etc. The fermentation in its production involved various biochemical processes and different lactic acid fermentation. Indigenous fermented food products enhances sustainable development in Nigeria through provision of employment opportunities, thus improving the livelihoods of the citizenry, poverty alleviation, empowerment initiatives, market improvement using simple, low-cost, traditional food processing techniques and enhanced food security providing regular income for the producers. Fermentation of indigenous fermented food also improves its organoleptic and preservative properties, adding to their nutritional quality. This paper outlines common indigenous fermented food products in Nigeria, microorganisms involved during the fermentation processes, their health benefits, emphasizing their sustainability towards National development and also the microbiological and biochemical changes during the fermentation processes.**

**Key words:** Indigenous, socio-economic, food security, national development.

### INTRODUCTION

Fermented foods are defined as those foods which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food (Campbell-Platt, 1987). They are described as palatable and wholesome and are generally appreciated for attributes, their pleasant flavors, aromas, textures, and improved cooking

and processing properties (Holzapfel, 2002). Fermented foods are produced in our homes, villages and also in small-scale food industries. Varieties of fermented foods are produced in developing countries, using traditional fermentation technology at the household level. The preparation of many indigenous fermented foods and beverages today remains a household-art. Traditional

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fermentation processes are typically uncontrolled and dependent on micro-organisms from the environment. Since the beginning of human civilization there has been an intimate relationship between man, his fare, and the fermentative activities of microorganisms. Traditional fermentation process is the most available and affordable food preservation method, which is of great economic importance to developing countries. Foods derived from fermentation are major constituents of the human diet all over the world (FAO/WHO, 1996). In some regions, mainly in African countries, fermentation plays important role in the nutrition of infants and young children as it is used for the preparation of complementary foods (Yasmine, 2000). Fermentation provides an economic means of preserving food and inhibiting the growth of pathogenic bacteria even under conditions where refrigeration or other means of safe storage are not available. It also enhances the nutritional quality of certain foods. In many parts of the world, particularly in Asia and in Africa, the technology has been traditionally used as a preservation method to ensure food safety (Yasmine, 2000). It preserves and enriches food, improves digestibility, enhances the taste and flavor of foods and is also easily accessible to all populace for its consumption. Fermentation also has the potential of enhancing food safety by controlling the growth and multiplication of a number of pathogens in foods (FAO, 2008). Thus, it makes an important contribution to human nutrition, particularly in developing countries, where economic problems pose a major barrier to ensuring food safety. The microorganisms responsible for the fermentation may be the microflora indigenously present on the substrate, or they may be added as starter cultures.

This paper aims to provide a review on the common indigenous fermented foods consumed in Nigeria, its sustainability towards national development and the microorganisms involved during the fermentation processes. As well as its health benefits of these food are summarized.

## **PROCESSES INVOLVED DURING FOOD FERMENTATION**

Fermentation is a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). The mechanism of food fermentation is essentially the conversion of carbohydrates to alcohols and carbon dioxide or organic acids by yeasts, bacteria, or a combination thereof, under anaerobic conditions. Lactic acid bacteria (LAB) are commonly involved in the fermentation of carbohydrate based substrates (Beukes et al., 2001). The two processes that are involved during the indigenous food fermentation are; Lactic acid fermentation (homolactic acid fermentation) and alcohol fermentation (heterolactic acid fermentation).

In fermentation, the substrate is only partially oxidized, ATP is formed exclusively by substrate-level phosphorylation, and oxygen is not needed. Lactic acid fermentation at the household level is a natural process brought about by lactic acid bacteria present in the raw food (WHO, 1989). In lactic acid fermented foods, the acidity is usually below pH 4. Most pathogenic microorganisms found in food cannot survive this low pH, hence, lactic acid fermentation of food has been found to reduce the risk of having pathogenic microorganisms growing in food (Sahlin, 1999).

## **COMMON INDIGENOUS FERMENTED FOODS IN NIGERIA**

Fermented foods contribute to about one-third of the diet worldwide (Campbell-Platt, 1987). They are described as palatable and wholesome and are generally appreciated for attributes: their pleasant flavors, aromas, textures, and improved cooking and processing properties (Holzapfel, 2002). Indigenous fermented foods have been prepared and consumed for thousands of years, and are strongly linked to culture and tradition (Sekar and Mariappan, 2005). They are processed through the activities of microorganisms. Indigenous food fermentation is one of the oldest biotechnology process in which microorganisms play an essential role in production and preservation. Many Indigenous fermented foods produced in Nigeria are fermented before consumption. The fermented foods in Nigeria can be classified into groups according to the substrates or raw materials employed (Odufa, 1985). These includes; *ogi*, *ogi baba*, *masa*, *pito*, *burukutu* (fermented products from maize, sorghum or millet), *gari*, *lafun*, *fufu* (fermented products from cassava), *iru*, *dawadawa* (fermented products from African locust beans and soya beans), *ogiri* (a fermented product from melon seeds), *nono* (fermented stimed cow milk/*wara-kishi*), *ugba* (fermented oil bean), *kunu zarki* (fermented millet), *palm wine*. These fermented foods are produced and processed using natural fermentation method.

## **MICROORGANISMS INVOLVED DURING THE FERMENTATION PROCESSES**

The indigenous natural fermentation takes place in a mixed colony of microorganisms such as moulds, bacteria and yeasts (William and Dennis, 2011). The microorganisms involved in African food fermentation are restricted to a few groups of yeasts and bacteria (Odufa, 1985). Microorganisms that are associated with the fermentation of different traditional fermented food products of Nigeria have being extensively studied by various researchers. The most common organisms responsible

for fermentation of foods are acid-forming bacteria such as genera lactic acid bacteria (LAB) which includes *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Corynebacterium*, *Enterococcus* and *Oenococcus*. Lactic acid bacteria that are important in food technology include those of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Leuconostoc* (Harrigan and McCance, 1990). Some yeast such as *Saccharomyces* and moulds such as *Penicillium*, *Aspergillus* and *Botrytis* are also involved during the fermentation process due to the lactic acid they produce (Wood and Holzapfel, 1995). Lactic acid bacteria (LAB) play a key role in safety and acceptability of fermented foods. Sanni and Adesulu (2013) reported reduction in pH during the fermentation of the maize meal for masa production and it was inferred that most bacteria including the pathogenic organisms do not survive in low pH environment and this imparts microbial safety as well as acceptability of the fermented food. Lactic acid bacteria are the most important group of microorganisms involved in the spontaneous or natural fermentation of foods (Steinkraus, 2002; Jakobsen and Lei, 2004). They are Gram positive, non-spore forming, catalase negative cocci or rods that are anaerobic, micro-aerophilic or aerotolerant (Wood and Holzapfel, 1995). Moulds are also involved during the food processing. Nearly all food fermentations are the result of more than one microorganism, either working together or in a sequence, but growth is generally initiated by bacteria, followed by yeasts and then moulds (FAO, 1998). Compounds formed during fermentation processes includes; organic acids (palmitic, pyruvic, lactic, acetic, propionic, malic, succinic, formic and butyric acids), alcohols (mainly ethanol) aldehydes and Ketones (acetaldehyde, acetoin, 2-methyl butanol) (Ari et al., 2012).

### IMPROVEMENT ON FERMENTED FOODS IN NIGERIA

Fermented foods are treasured as major dietary constituents in numerous developing countries because of their keeping quality, undue ambient conditions enhancing nutritional quality and digestibility, improve food safety, and are traditionally acceptable and accessible (Holzapfel, 2002, Rolle and Satin, 2002). Indigenous fermented products, according to the mechanism that occur during the fermentation helps to promote safety of foods. However, the final product can be risky resulting from the poor hygienic conditions during processing. Optimization of the fermentation process will result in production of quality, safe and acceptable fermented foods and these can be carried out by: Application of appropriate starter culture, back-slopping, raw material development, enhancement of fermentation processes, application of HACCP system and Good Manufacturing Practice, development of better equipment and development of standard for commercialization.

### HEALTH BENEFITS OF INDIGENOUS FERMENTED FOODS

A number of foods especially cereals are poor in nutritional value, which constitute the main staple diet of the low income populations (Chelule et al., 2010). However, Lactic Acid Bacteria fermentation has been shown to improve the nutritional value and digestibility of these foods (Nout, 2009). The enzymes including amylases, proteases, phytases and lipases, modify the primary food products through hydrolysis of polysaccharides, proteins, phytates and lipids respectively (Adeyemi and Muhammad, 2008). The quantity and quality of the food proteins as expressed by biological value, and often the content of water soluble vitamins is generally increased, while the antinutrient factors (ANFs) such as phytic acid and tannins in food decline during fermentation leading to increased bioavailability of minerals such as calcium, phosphorus, zinc, iron, amino acids and simple sugars (Santos et al., 2008; Soetan and Oyewole, 2009; Murwan and Ali, 2011). Some of the microorganisms involved in traditional fermentations present beneficial effects in human health; they cause reduction of toxin in food, with the binding of aflatoxins by yeast of the genus *Saccharomyces*. Lactic acid bacteria in fermentation detoxified toxins and are more advantageous, in that it is a milder method which preserves the nutritive value and flavor of foods (Chelule et al., 2010). Fermentation irreversibly degrades mycotoxins without adversely affecting the nutritional value of the food (Ari et al., 2012).

Many of the fermented products consumed by different ethnic groups have therapeutic values, some of the most widely known are fermented milks (that is, yoghurt, curds) which contain high concentrations of probiotic bacteria that can lower the cholesterol level (Jyoti, 2010), Improvement of nutrients absorption and digestion, restores the balance of bacteria in the gut to hinder constipation, abdominal cramps, asthma, allergies, lactose and gluten intolerance (Abdel et al., 2009). The slurries of carbohydrate based fermented Nigerian foods such as ogi, fufu and wara have been known to exhibit health promoting properties such as control of gastroenteritis in animals and human (Aderiye et al., 2007; Olukoya et al., 2011). Some microorganisms involved during the fermentation also produces antimicrobial products that lead to safe and long storing of foods (Steinkraus, 2002).

### ENHANCING SUSTAINABILITY OF INDIGENOUS FERMENTED FOOD PRODUCTS IN NIGERIA

Fermented food products can play an important role contributing to the livelihoods of rural and periurban dwellers alike, through enhanced food security, and income generation via a valuable small scale enterprise option (FAO, 2011). Fermentation activities plays significant role towards contributing to the livelihoods of women, the disabled and

landless poor who, with appropriate training and access to inputs, can increase their independence and self-esteem through income generation (FAO, 1998). Fermented food products play an important socio-economic role in Nigeria. In enhancing sustainability of indigenous fermented food products in Nigeria, there is a need to overcome constraints that may cause set back in the socio-economic status of the populace and stipulating standards that will help every citizenry to alleviate poverty and other vices that may pose threat to the National economy. Sustainable development is enhanced with these suitable approaches;

### **Eradication of food shortage**

Fermented foods constitute a major portion of peoples' diets all over the world and make a major contribution to dietary staples in numerous countries across Africa, and the world at large, providing 20 - 40% of the total food supply (Abdel et al., 2009). Small-scale fermentation technologies contribute substantially to food security and nutrition, particularly in regions that are vulnerable to food shortages (FAO, 1998), this thus contributes to sustainable development of the country as little is spent on imported foods.

### **Food security**

Fermentation as a means of food preservation and preparation has the potential of meeting the Nigerian's food supply demand if it is mechanized and industrialized. This is achieved through improved food preservation method, increasing the range of raw materials that can be used to produce edible food products in large quantity and removing anti-nutritional factors to make food safe to eat. Indigenous fermented foods promote and improve food processing and preservation at all levels of operation, which are essential component of national strategic plans for food security aim at achieving national food security (Adeyemi and Muhammad, 2008).

### **Employment benefits**

Fermentation enterprises are employers of many millions of people in the world (Elaine and Danilo, 2011). They are considered to be industrial set ups process, prepare, package, market and in some cases brand products and employ many thousands of people (FAO, 2011). Majority of employment comes from small scale fermentation enterprises. Traditional and small-scale fermentation enterprises have a good employment record, especially in remote areas, even where access to sophisticated equipment is limited. As a result of increased and continual popularity of fermented products', such small-scale enterprises potentially have the capacity of generating even

more widespread employment options (FAO, 2011). By generating employment opportunities in the rural areas, small scale food industries reduce rural-urban migration and the associated social problems (Aworh, 2008). In Japan and Latin America, fermented food forms important sectors of their economy, where more than 560 000 tonnes of *miso* (fermented legume product) is produced yearly to form fermented cereal products, alcoholic drinks, and fermented milk products (FAO, 1998), fermentation of foods thereby provides global importance of employment.

### **Youth empowerment initiatives**

Youths in developing nations are the sect of people who have great strength and potentials in them and if they are adequately empowered they will produce their best and indirectly providing employment benefits to the unemployed youths, this will greatly increase and promote sustainable growth. They will also find ready employment on their door step and will have the opportunity to increase their knowledge in fermentation practices, thus becoming competent and skilled in a profession. Organizations can also help with training, capacity building and improving fermentation techniques, thereby empowering them. Moreover if subject to training in fermentation processes, process management, quality control and business matters, their capacity will increase as new knowledge will be gained and will have more transferable skills than the crude methods employed so far.

### **Reduction in mortality rate**

Fermentation prevents the enlargement of pancreas. Since fermented foods are rich in enzymes, there is high reduction in mortality rate. "Eating an enzyme-rich diet decreases the load on pancreas, preserving the body's own natural enzyme potential, thereby reducing the risk of chronic diseases". Eat more raw food, fermented food and living food (Kristen, 2011).

### **Industrialization**

Indigenous fermented foods production provides opportunities for generation of income through employment and thereby promoting industrial development in Nigeria. Small-scale food industries that involve lower capital investment and that rely on traditional food processing technologies are crucial to rural development in Nigeria (Aworh, 2008). Industrialization brings about economic development of any nation; this will have concerted effect on the sustenance of average citizenry of the nation.

### **Poverty alleviation**

Poverty alleviation scheme set up by governments and agric banks in form of loans and credits has helped to

alleviate poverty to a very low level among the populace and has provided a source of income, it also contributes to the food security of the nation. Government setting up resource workers to organize programmes and enlightening the farmers on the potential opportunities of earning income from providing certain fermented products, this will enable better production planning, less waste, reduction in costs and potentially making only products that have a good demand and thus increasing the likelihood of selling and earning income. Moreover by meeting with customers regularly can help develop a farmer's local sales network playing a beneficial role in poverty alleviation and economical development of the nation.

### Market improvement

The general level of market development in areas where fermented goods are promoted is an important factor determining their market potential. Good understanding of the fermentation process usually based on traditional knowledge but may also be acquired or improved through external support from governments and research institutes thus allow producers to provide consistent and predictable quantities and qualities of fermented products, thereby attracting buyers more easily. Fermentation activities undertaken represent an important economic opportunity, earnings made can provide for family needs and savings.

### Food supplement

The locally fermented foods serve as food supplement like the use of ogi as a weaning food in Southern Nigeria to supplement breastfeeding (Falana et al., 2011). The fermented foods made available the diet required in human body (Egberere, 2008). Traditional fermented protein-rich foods offer excellent opportunities for improving the diets of people in tropical countries providing rich source of starch, vitamins, proteins and minerals (Oladejo and Adetunji, 2012).

### Food availability

They are vital to reducing post-harvest food losses and increasing food availability (Aworh, 2008). Fermented foods are of great significant because they provide and preserve vast quantities of nutritious foods in a wide diversity of flavours, aromas and textures which enrich the human diet (Yabaya, 2008).

### CONCLUSION

Several indigenous fermented food products are processed in Nigeria. The fermentation technology practices

by the traditional people shows a strong correlation of these people with nature and the assessment of microbial benefits. The rich microbial diversity in various sources of fermented foods reflects that the indigenous people have been harnessing indigenous microbiota for spontaneous fermentation. Fermentation technology practices contribute to subsistence of regional economy and prove as a boost to the livelihood of the rural people. These traditional methods of fermentation and preservation can be commercialized and productivity can be maximized if contributions in terms of support are provided by various governing bodies and institutes. As an appropriate food preservation technology, the economic and social benefit of applying small-scale fermentation in food preservation contributes to sustainable development. Fermentation however, requires very little sophisticated equipment, either to undertake or subsequently store the fermented product, and has had a major impact on nutritional habits, traditions, and culture. The development of appropriate approaches aimed at enhancing the quality of indigenous Nigeria foods will be indispensable for the growth and survival of the food industry.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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## Full Length Research Paper

## Mode of vertical transmission of *Salmonella enterica* sub. *enterica* serovar Pullorum in chickens

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The mode of vertical transmission was studied with local isolate of *Salmonella pullorum* in hens and cocks. Twenty (20) hens and five cocks were experimentally infected by the oral route with  $2 \times 10^7$  (CFU) dose of *S. pullorum* organisms at 21 weeks of age in hens and 29 weeks in cocks and in control (20 hens and five cocks) no bacteria was given. The used methods were reisolation of *S. pullorum* from different organs, blood, eggs and newly hatched chicks, and detection of *S. pullorum* by PCR from testicular tissue at different time intervals of experimental period. Five birds (four hens and one cock) randomly selected and sacrificed on 6 h before inoculation (BI) and one, two, three and four weeks of post-infection (PI) from infected and control group. *S. pullorum* was reisolated from 50% eggs of experimentally infected hens. Twenty percent hatchability was lost due to experimental *S. pullorum* infection. Piped chicks were 20% and embryo mortality was 15%. *S. pullorum* was isolated from 66.66% chicks. Seventy five percent testes were positive for *S. pullorum* by culture and biochemical test. *S. pullorum* was detected by PCR at one to three weeks PI from testicular tissues. It was clear that after oral route of infection with infective dose of *S. pullorum*, the bacteria invaded digestive epithelia and ultimately entered into blood inducing bacteremia and ultimately infected different organs and produced pathological lesions. It was also confirmed that the bacteria invaded ovary and egg follicles, and this infection persisted in ovary and egg follicles and transmitted into laid eggs then to hatched chicks.

**Key words:** Vertical transmission, *Salmonella pullorum*, chickens.

### INTRODUCTION

Pullorum disease (PD) is an acute, infectious, and fatal disease of chicks causing mortality as well as results in persistent infection and vertical transmission in layer birds (Wray and Davies, 2001; Ramasamy et al., 2012). PD in growing and mature fowl is characterized by a sudden drop in feed consumption, with ruffled feathers, and pale and shrunk combs. Other signs in laying hens are characterized by a drop in egg production, decreased

fertility, and diminished hatchability. The prominent signs of PD are anorexia, diarrhoea, depression, and dehydration (Chauhan and Roy, 1996; Haider et al., 2003; Hossain et al., 2006).

Pullorum disease may be transmitted horizontally and vertically. Vertical transmission may result from contamination of the ovum following ovulation, but localization of *Salmonella pullorum* in the ovules before ovulation

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also likely and probably constitutes the chief mode of transmission (Wigely et al., 2005). Embryonic dead and chick mortality in PD due to vertical transmission are the major problems, and it causes great economic losses in the poultry industry of Bangladesh. However, in Bangladesh no systematic investigation has been performed on the experimental vertical transmission of PD in laying hens. Therefore, this investigation is designed to correlate among oral routes of infection with *S. pullorum*, ovarian follicle infection, reisolation of *S. pullorum* from experimentally inoculated ovarian follicles of infected hens and eggs laid by them, and pathological lesions of ovarian follicles of experimentally inoculated infected hens. Findings obtained from this investigation would be useful for control and eradication measures of PD spread by vertical route of transmission in Bangladesh.

PD causes great economic losses every year in poultry farms and it has also zoonotic importance. The diseases can spread via meat and eggs. For exporting the poultry meat and eggs, salmonellae should be free in Bangladesh.

A few investigations on natural cases of *Salmonella* infections have been completed in Bangladesh using the methods of necropsy, histopathology and isolation of bacteria by culture; staining and sugar fermentation tests (Ahmed et al., 2008; Haider et al., 2008; Islam et al., 2006). However no investigations have been performed by locally isolated *S. pullorum* organisms in experimental pathogenesis, pathology and vertical transmission in chickens. For this reason, the present study was taken first time for better diagnosis, prevention and control of this economically important pullorum disease in Bangladesh.

## MATERIALS AND METHODS

The chickens of different age group were purchased from Nourish Hatchery Ltd, Shreepur, Gazipur and reared in different poultry sheds of the Department of Pathology and poultry units of the Department of Microbiology and Hygiene.

### Experimental hens and cocks

A total of 40 pullets (*Salmonella pullorum* seronegative) of Isa Brown breed of 18 weeks were purchased from Nourish Hatchery Ltd., Shreepur, Gazipur, Bangladesh and 10 cocks (*Salmonella pullorum* seronegative) of RIR (Road Island Red) breed of 26 weeks old were taken from BAU Poultry farm. The birds were vaccinated against Marek's Disease, Infectious Bursal Disease, Infectious Bronchitis, Fowl Pox and Newcastle Disease obtained from Intervet, Holland. The birds were divided into two groups in which one group remained as control. At the beginning of laying the pullets were called as hens.

### Bacterial infection

25 (20 hens at the age of 21 weeks and five cocks at the age of 29

weeks) birds were experimentally infected by the oral route with  $2 \times 10^7$  (CFU, Colony Forming Unit) dose of *Salmonella enterica* sub. *enterica* serovar Pullorum (Isolate No. 5) organisms in 0.5 ml broth culture with 0.5 ml of sterile phosphate buffer saline (PBS), pH 7.2, using sterile syringe (Roy et al., 2001; Wigley et al., 2005). Control birds were given only 0.5 ml broth without bacteria with 0.5 ml of PBS.

### Samples collection

Five (five hens and one cock) birds in each case were randomly selected and sacrificed 6 h before inoculation and one, two, three and four weeks PI. A total of 25 (20 hens and 5 cocks) birds were used for the control group, and necropsies were carried out in similar a similar matter as the infection groups. Different types of samples were collected as described earlier. Eggs, ova and parts of female reproductive organs were collected for the isolation of microorga-nisms (Wigley et al., 2005).

### Re-isolation of *Salmonella pullorum*

Collected samples (crop, liver, lung, heart, duodenum, cecum, kidney, bile and spleen) were weighed and placed in a tube containing 1 ml of sterile phosphate - buffered saline (PBS) solution. The colony-forming units of *S. pullorum* were counted using standard microbiological methods (Haider et al., 2008; Haider et al., 2012).

For each bird in each group of 6 h before inoculation and 1, 2, 3 and four weeks post-infection, 1 ml of blood was collected and *S. pullorum* was re-isolated using previously described methods (Haider et al., 2008; Haider et al., 2012). *S. pullorum* was reisolated from ovary, ovarian follicle, oviduct, uterus, vagina and testis as described previously (Wigely et al., 2005). Laid eggs were collected and soaked in Lugol's iodine for 20 min. The presence of *S. pullorum* was confirmed by standard procedures (Cheesbrough, 2000; Haider et al., 2003; Haider et al., 2012).

### Hatching of eggs

Standard procedure was followed for preparation of incubator and setting eggs in the incubator. The candling of eggs was also done at 10 and 18 days of incubation with the help of electric Candler for dead embryos (Carol and Gregg, 2002):

The unhatched eggs were broken up to confirm the death of embryos.

The chicks that piped but did not come out of the shell were considered piped chicks.

The chicks that came out of the shell on 21 days of incubation that were recorded as hatched chicks.

Fertility of eggs was calculated by using the following formula:

$$\text{Fertility (\%)} = \frac{\text{Total number of fertile eggs}}{\text{Total number of eggs set}} \times 100$$

Hatchability of eggs was calculated on the basis of egg set and on fertile eggs, and these were calculated by the following formulae (Carol and Gregg, 2002):

$$\text{Hatchability on eggs set (\%)} = \frac{\text{Total number of chicks hatched}}{\text{Total number of eggs set}} \times 100$$

**Table 1.** Average number of CFU/g of isolated and identified of *Salmonella pullorum* from different organs of experimentally infected hens.

Organ	BI 6 h	PI 1	PI 2	PI 3	PI 4
1. Crop	00(0/4)	$58.72 \times 10^4(4/4)$	$25.81 \times 10^3(4/4)$	$25.68 \times 10^2(3/4)$	$14.96 \times 10^1(2/4)$
2. Liver	00(0/4)	$64.58 \times 10^5(4/4)$	$65.79 \times 10^4(4/4)$	$55.98 \times 10^3(4/4)$	$44.28 \times 10^3(3/4)$
3. Heart	00(0/4)	$21.34 \times 10^3(4/4)$	$40.16 \times 10^3(4/4)$	$48.66 \times 10^2(3/4)$	$14.71 \times 10^2(3/4)$
4. Lungs	00(0/4)	$44.85 \times 10^4(4/4)$	$39.02.2 \times 10^4(4/4)$	$66.95 \times 10^3(4/4)$	$24.26 \times 10^2(4/4)$
5. Duodenum	00(0/4)	$20.98 \times 10^5(4/4)$	$47.51 \times 10^4(4/4)$	$36.54 \times 10^4(4/4)$	$43.56 \times 10^3(4/4)$
6. Cecum	00(0/4)	$81.11 \times 10^5(4/4)$	$73.93 \times 10^5(4/4)$	$64.47 \times 10^4(4/4)$	$80.77 \times 10^3(4/4)$
7. Bile	00(0/4)	$47.88 \times 10^3(2/4)$	$14.29 \times 10^3(1/4)$	00(0/4)	00(0/4)
8. Kidney	00(0/4)	$39.72 \times 10^3(4/4)$	$13.58 \times 10^3(4/4)$	$18.84 \times 10^2(2/4)$	$19.26 \times 10^1(2/4)$
9. Spleen	00(0/4)	$89.25 \times 10^5(4/4)$	$93.34 \times 10^5(4/4)$	$62.21 \times 10^3(4/4)$	$51.25 \times 10^3(4/4)$

$$\text{Hatchability on fertile eggs (\%)} = \frac{\text{Total number of chicks hatched}}{\text{Total number of fertile eggs}} \times 100$$

Embryonic mortality was calculated by the following formula

$$\text{Embryonic mortality (\%)} = \frac{\text{Total number of dead embryos}}{\text{Total number of fertile eggs}} \times 100$$

*S. pullorum* was re-isolated from liver, lungs, heart, ceca and yolk materials from newly hatched chicks and dead embryos as described by Wigely et al., 2005.

#### Detection of *Salmonella pullorum* by PCR

Testes were collected in sterile poly bag with PBS at different time intervals. Testicular tissue was used for the detection of microorganisms by PCR (Rahn et al., 1992).

## RESULTS

Re-isolated *S. pullorum* produced pink colour colonies on BGA, and CFU/g of tissues was counted and recorded. *S. pullorum* showed red - pink - white opaque coloured colonies surrounded by brilliant red zones in BGA. In Gram's staining, the morphology of the isolated bacteria was small, rod shape, Gram negative and single or paired in arrangement. *S. pullorum* produced an alkaline (red) slant and acid (yellow) butt, with gas bubbles in the agar and a blackening due to H<sub>2</sub>S production observed the acid reaction of the butt in TSI agar. *S. pullorum* showed lysine decarboxylation, with a deeper purple (alkaline) slant and alkaline or neutral butt with slight blackening due to H<sub>2</sub>S production in LI agar. The isolated organisms fermented dextrose, manitol and xylose with gas production and did not ferment lactose, sucrose, dulcitol, inositol and maltose. The organisms were positive to MR test and were negative to indole and VP test. Limited movement was observed in the isolated organisms.

#### Re-isolation of *Salmonella pullorum* from different organs of hens

Reisolation of *S. pullorum* from different organs was variable in different time schedule (Table 1). Of the total tissue collected, 93.75% liver, 100% lungs, 100% duodenum, 100% ceca and 100% spleen were positive for *S. pullorum* at 1 to 4 weeks PI. The *S. pullorum* was re-isolated from 81.25% crop, 87.5% heart, 18.75% bile and 75% kidney throughout the study period. The highest number of *S. pullorum* re-isolated was  $64.58 \times 10^5$  in liver at 1 week PI and the lowest was  $14.96 \times 10^1$  in crop at 4 weeks PI. The average numbers of CFU/g of re-isolated *S. pullorum* from different time intervals are shown in Table 1. Control group was free from *S. pullorum* in culture during the study period.

#### Reisolation of *Salmonella pullorum* from blood

The average number of CFU/ml of *S. pullorum* re-isolated from blood shown in Table 2. The blood sample of four hens out of four (4/4) at 1 and 2 weeks PI, three hens out of four (3/4) and one hens out of four (1/4) at 3 weeks and four weeks PI, respectively, were positive for *S. pullorum*. The highest number of CFU/ml was  $13.55 \times 10^3$  at 1 PI and the lowest was  $13 \times 10^2$  at 4 weeks PI. No *S. pullorum* was found in the control group. Colony characters and results of biochemical tests of re-isolated *S. pullorum* were found similar which were described earlier.

#### Re-isolation of *S. pullorum* from female reproductive organs

*Salmonella pullorum* was re-isolated from ovary (100%), ovarian follicle (100%), oviduct (68.75%), uterus (56.25%) and vagina (75%) of female reproductive organs after experimental infection (Table 3). No *S. pullorum* was found in control group hens. Re-isolated *S.*



**Table 2.** Re-isolation and average number of CFU/ml of isolated and identified of *Salmonella pullorum* from blood of experimentally infected hens.

Time schedule of PI	Infection group		Control group	
	Number	Mean CFU	Number	Mean CFU
BI 6 h	0/4	00	0/4	00
PI 1	4/4	$13.55 \times 10^3$	0/4	00
PI 2	4/4	$8.43 \times 10^3$	0/4	00
PI 3	3/4	$33.76 \times 10^2$	0/4	00
PI 4	1/4	$13 \times 10^2$	0/4	00

Percentage calculated from 1 PI to 4 weeks PI

**Table 3.** Average number of CFU/g of isolated and identified *Salmonella pullorum* from female reproductive organs in experimentally infected hens.

Organ	BI 6 h	PI 1	PI 2w	PI 3	PI 4	Total (%)
Ovary	00(0/4)	$41.05 \times 10^5(4/4)$	$64.6 \times 10^4(4/4)$	$33.05 \times 10^3(4/4)$	$40.93 \times 10^4(4/4)$	100
Ovarian follicle	00(0/4)	$67.75 \times 10^4(4/4)$	$47.61 \times 10^4(4/4)$	$31.67 \times 10^3(4/4)$	$49.5 \times 10^3(4/4)$	100
Oviduct	00(0/4)	$39.65 \times 10^4(4/4)$	$25.17 \times 10^3(3/4)$	$40.67 \times 10^3(2/4)$	$27.54 \times 10^3(2/4)$	68.75
Uterus	00(0/4)	$40.11 \times 10^5(3/4)$	$14.36 \times 10^3(3/4)$	$19.16 \times 10^3(2/4)$	$30.81 \times 10^3(1/4)$	56.25
Vagina	00(0/4)	$51.15 \times 10^3(4/4)$	$07.2 \times 10^3(4/4)$	$43.12 \times 10^3(3/4)$	$13.13 \times 10^3(1/4)$	75

Percentage calculated from 1 PI to 4 weeks PI.

**Table 4.** Average number of CFU/g of isolated and identified *Salmonella pullorum* from different organs of cocks after experimental infection.

Organ	BI 6 h	PI 1	PI 2w	PI 3	PI 4	Total (%)
Liver	00(0/1)	$69.83 \times 10^3(1/1)$	$158.62 \times 10^3(1/1)$	$37.05 \times 10^3(1/1)$	$40.93 \times 10^2(1/1)$	100
Lung	00(0/1)	$60.89 \times 10^3(1/1)$	$86.90 \times 10^3(1/1)$	$31.67 \times 10^3(1/1)$	$49.5 \times 10^2(1/1)$	100
Heart	00(0/1)	$12.24 \times 10^3(1/1)$	$23.31 \times 10^3(1/1)$	$41.67 \times 10^2(1/1)$	00(0/1)	75
Spleen	00(0/1)	$105.41 \times 10^3(1/1)$	$163.36 \times 10^3(1/1)$	$89.16 \times 10^3(1/1)$	$30.81 \times 10^2(1/1)$	100
Cecum	00(0/1)	$236.88 \times 10^3(1/1)$	$118.68 \times 10^3(1/1)$	$103.12 \times 10^3(1/1)$	$83.13 \times 10^3(1/1)$	100
Testis	00(0/1)	$35 \times 10^2(1/1)$	$16.4 \times 10^3(1/1)$	$19.32 \times 10^2(1/1)$	00(0/1)	75

*pullorum* produced pink colour colonies on BGA, and CFU/g of tissues was counted and recorded.

#### Re-isolation of *S. pullorum* from different organs of cocks

Re-isolation rate of *S. pullorum* from different organs was variable in different time schedules (Table 4). 100% liver, 100% lungs, 75% heart, 100% cecum, 100% spleen and 75% testes were positive for *S. pullorum* at 1 to 4 weeks PI. Control group was free from *S. pullorum* in culture during the study period. Re-isolated *S. pullorum* produced pink colour colonies on BGA, and CFU/g of tissues was counted and recorded.

#### Re-isolation of *Salmonella pullorum* from laid eggs

Average number of CFU/g of isolated and identified of *S.*

*pullorum* from eggs of experimentally infected hens are showed in Table 5. Isolation rate of *S. pullorum* from outer shell of laid eggs was 95%, the highest positive while, the second highest was 50% in the egg yolk. The lowest isolation rate of *S. pullorum* was in inner shell and egg albumin, which were 45 and 35%, respectively. Throughout the study period *S. pullorum* was re-isolated from 50% of the laid eggs and no *S. pullorum* was found in control hen's laid eggs. Re isolated *S. pullorum* produced pink colour colonies on BGA, and CFU/g of tissues was counted and recorded.

#### Hatching of eggs

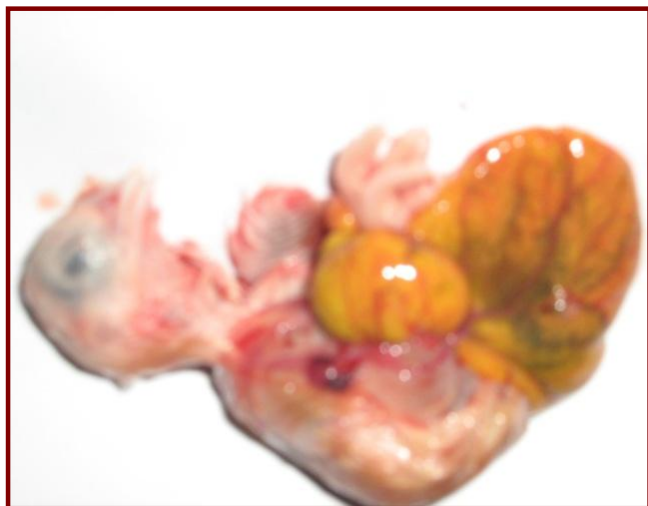
Hatching information of eggs after experimental infection with *S. pullorum* is shown in the Table 6. Fertility was 65% in the infection group and 85% in control group

**Table 5.** Average number of CFU/g of isolated and identified *Salmonella pullorum* from eggs of experimentally infected hens.

Source	BI 6 h	PI 1	PI 2w	PI 3	PI 4	Total (%)
Outer shell	00(0/5)	$11.1 \times 10^6(4/5)$	$05.52 \times 10^6(5/5)$	$04.38 \times 10^3(5/5)$	$17.82 \times 10^3(5/5)$	95
Inner shell	00(0/5)	$12.59 \times 0^3(2/5)$	$01.78 \times 10^3(3/5)$	$01.36 \times 10^3(2/5)$	$04.26 \times 10^3(2/5)$	45
Egg albumin	00(0/5)	$41.42 \times 0^3(2/5)$	$01.99 \times 10^3(2/5)$	$10.69 \times 10^3(2/5)$	$13.82 \times 10^2(1/5)$	35
Egg yolk	00(0/5)	$44.1 \times 10^3(3/5)$	$10.3 \times 10^3(3/5)$	$78.5 \times 10^5(2/5)$	$24.36 \times 10^3(2/5)$	50

**Table 6.** Hatching indices of chicks after experimental infection with *Salmonella pullorum* (n=20).

Hatching index	Infection group (%)	Control group (%)
1. Fertility	65	85
2. Hatchability		
a. Hatchability on eggs set	50	85
b. Hatchability on fertile eggs	77	100
3. Embryonic mortality	15	00
4. Dead-in- shell	15	00
5. Piped chicks	20	05

**Figure 1.** Showing the embryonic death of chicks with unabsorbed and coagulated egg-yolk after experimental infection with *S. Pullourm*.**Figure 2.** Showing the piped chicks after experimental infection with *S. Pullourm*.

hen's laid eggs. Hatchability of eggs set and hatchability of fertile eggs were 50 and 77%, respectively in infected eggs.

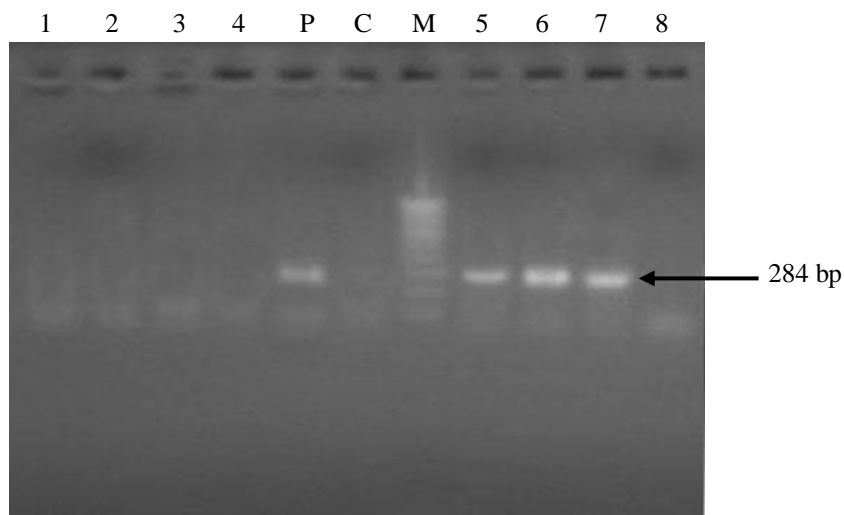
Hatchability of 100% on fertile eggs was found in the control group. The embryonic mortality (Figure 1), dead-in-shell and piped chicks (Figure 2) were 15, 15 and 20%, respectively. No embryonic mortality, no dead-in-shell and 5% piped chicks were found in the control group eggs.

#### Re-isolation of *S. pullorum* from different organs of newly hatched chicks and dead embryos

*S. pullorum* was re-isolated in 66.66% of liver, lungs, ceca and yolk materials, and 55.55% of heart from newly hatched chicks and dead embryos, respectively (Table 7). The highest CFU of *S. pullorum* was  $59.23 \times 10^6$  re-isolated from yolk materials. No *S. pullorum* was found in control chicks. Re-isolated *S. pullorum* produced pink

**Table 7.** Average number of CFU/g of re-isolated and identified of *S. pullorum* from different organs of newly hatched chicks of infected group (n=9) and control group (n=10).

Organ	Control group	% Isolation	Infection group	% Isolation
Liver	00 (0/10)	00	$49.11 \times 10^4$ (6/9)	66.66
Lungs	00 (0/10)	00	$33.28 \times 10^4$ (6/9)	66.66
Heart	00 (0/10)	00	$17.9 \times 10^4$ (5/9)	55.55
Ceca	00 (0/10)	00	$54.4 \times 10^6$ (6/9)	66.66
Yolk materials	00 (0/10)	00	$59.23 \times 10^6$ (6/9)	66.66



**Figure 3.** Electrophoresis of amplified samples of testes in control and infected cocks specific for *Salmonella pullorum* on 1.5% agarose gel. No bands are seen in lanes 1 to 4 from testes in the control group of cocks; lane P showing the 284-bp band as a positive control; lane C showing no band as a negative control; lanes 5 to 7 showing 284-bp bands from testes of infected cocks at 1 to 3 weeks PI, and lane 8 showing no band at 4 weeks PI from testes of infected cocks. Lane M showing DNA molecular Mass marker (100).

colour colonies on BGA, and CFU/g of tissues was counted and recorded.

#### Detection of *Salmonella pullorum* by PCR

*S. pullorum* was detected by PCR at 1 PI to 3 weeks PI from testicular tissue of infected cocks (Figure 3). No amplification was seen at 4 weeks PI and in the control group of cocks.

#### DISCUSSION

*Salmonella enterica* serovar Pullorum causes persistent infections in laying hens. Splenic macrophages are the main site of persistence. At sexual maturity, numbers of bacteria increase and spread to the reproductive tract which this may result in vertical transmission to eggs or

chicks (Wigley et al., 2005). Eggs transmission may result from contamination of the ovum following ovulation, but localization of *S. pullorum* in the oviducts before ovulation is also likely and probably constitutes the main mode of transmission (Shivaprasad, 1997). Transmission through eggshell penetration by *S. pullorum* is also reported (Shivaprasad, 1997; Williams et al., 1968). In this investigation, localization of *S. pullorum* in specific cell types was not detected by immunohistochemistry. The other findings of the present study agree with previous reports (Shivaprasad 1997; Wigley et al., 2005; Williams et al., 1968).

In this study, the birds experimentally infected at 18 weeks through orally with  $2 \times 10^7$  *S. pullorum* and the re-isolation rate was 50% in laid eggs during the study period. Wigley et al. (2001), found in commercial brown-egg layers infected orally  $1 \times 10^9$  organisms of *S. pullorum* at 1 of age laid eggs 60% positive for *S. pullorum* from 18 weeks PI to 42 weeks PI but the average

positive was 6.5%. In the present study, re-isolation of *S. pullorum* was highest at 2 PI and decreased gradually at 3 weeks PI to onward while Okamura et al. (2001) recovered *S. enteritidis* increased from 50% at 4 days PI to 100% 7 days PI and viable count also increased. In the present study, 50% laid eggs was infected with *S. pullorum* but Chauhan and Roy (1996) reported 34% infected laid eggs; Shivaprasad (1997) reported 33% infected laid eggs; and Wary and Davies (2001) only a small percentage of the eggs is also likely to be infected with *S. pullorum*.

In the present study, *S. pullorum* were re-isolated from different reproductive organs of male and female, eggs and newly hatched chicks at experimental PI at different time intervals. *S. pullorum* was re-isolated from ovary (100%), ovarian follicle (100%), oviduct (68.75%), uterus (56.25%) and vagina (75%) of female reproductive organs after experimental infection at 21 weeks of age with  $2 \times 10^7$  CFU of *S. pullorum* in this study. While Kinde et al. (2000) recorded ovary (58%) and oviduct (42%) positive with the field isolate of *Salmonella enteritidis* phage type 4 and Okamura et al. (2001) recovered *S. enteritidis*, *S. Typhimurium* and *Salmonella Hadar* from the ovary 100, 40 and 20%, and from follicles 87.5, 10, and 13.36%, respectively at 4 days PI. *Salmonella* serovar Pullorum was recovered from 75% reproductive tracts of chickens, 80% ovary and 60% oviduct at 18 weeks PI, and 37.5% ovary and 12.5% oviduct at 22 weeks PI by Wigley et al. (2005). Ovaries (100%) and oviducts (80%) were found positive for *S. pullorum* at 20 weeks PI (Wigley et al., 2001). Michailidis et al. (2012) and Anastasiadou et al. (2013) quantified antimicrobial peptides avian  $\beta$ -defesins (Av $\beta$ Ds) in chicken ovary and vagina, respectively, during sexual maturation and *Salmonella* infection using real-time PCR.

Trampel (2001) reported 55% reduction of hatchability in clinical outbreaks of pullorum disease (PD) in laying hens. In the present study 23% reduction of hatchability on fertile eggs was found in infection group in comparison to control group. It is indicating that PD has the effects on hatchability but the exact mechanisms cannot be explained from this experiment. In this study, 20% fertility was reduced in comparison to control group while other authors (Shivaprasad, 1997; Trampel, 2001; Wray and Davies, 2001) reported the reduction in some percentage of fertility in PD.

Chauhan and Roy (1996), Shivaprasad (1997), Trampel (2001), Withanage et al. (2004) and Wray and Davies (2001) speculated that the infection to chicks comes from the infected eggs laid by a carrier hen. In the incubator, the hatched diseased chicks spread infection to other healthy chicks. In the present study, *S. pullorum* were re-isolated from hatched chicks of infection group of liver (66.66%), lung (66.66%), heart (55.55%), cecum (66.66%) and yolk materials (66.66%). It may be speculated that hatched chicks received infection from infected eggs laid by infected hens.

In the present study, *S. pullorum* was re-isolated from 75% testes of cocks but Wigley et al. (2005) could not re-isolate *S. pullorum* in the testes of male birds. In this experiments, it is cleared that after oral route of infection with infective dose of *S. pullorum*, the bacteria invades digestive epithelia and ultimately enters into blood causing bacteremia which is corresponded with the finding of Haider et al. (2012). From blood, bacteria are seeded into cells and tissues of different organs such as liver, lung, spleen, kidney, different parts of reproductive tracts of hens and male testes. It is also confirmed that the bacteria invades ovary and egg follicles, and this infection persists in ovary and egg follicles and transmits into laid eggs then to hatched chicks. In this study vertical transmission is known in chickens. In future for the control of *Salmonella* infections in poultry, vaccine production and sequencing of vaccine candidate in association with phylogenetic analysis of circulating *Salmonella* organisms would be performed in Bangladesh.

### Conflict of interests

The author(s) have not declared any conflict of interests.

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