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*Full Length Research Paper*

# Seasonal emergence of swine erysipelas in hilly state Nagaland, Northeast India

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**Seasonal outbreaks of swine erysipelas have been reported in back yard pig farms in the Phek district of Nagaland, India. The alpha haemolytic isolate of *Erysipelothrix rhusiopathiae* was recovered on blood agar from the clinical samples. The organisms were confirmed microscopically, biochemical analysis as well as by polymerase chain reaction (PCR) amplification of 16S rRNA gene and sequence analysis. These Nagaland isolates (KT160358, KT160359) were closely related to the type spp. *E. rhusiopathiae* in phylogenetic analysis and forms the same clad with Chineese isolates of swine and murine origin indicating an epidemiological link. The isolates were found to be most sensitive to oxytetracycline and responded to treatment. Swine erysipelas occurred in Phek district in a season due to sudden change of weather and temperature. Pigs exposed to such predisposing factors probably favoured to propagation of already persisted organisms in pigs. This is the first confirmed case of *E. rhusiopathiae* infection from the NE states of Nagaland, India.**

**Key words:** Swine erysipelas, *Erysipelothrix rhusiopathiae*, pig, polymerase chain reaction (PCR) Nagaland, India, Oxytetracycline.

## INTRODUCTION

*Erysipelothrix rhusiopathiae,* belonging to the family *Erysipelotrichaceae,* is a non-motile, Gram-positive, non-sporulating, non-acid-fast organism distributed worldwide affecting wide variety of vertebrate and invertebrate species including man (Reboli and Farrar, 1989). Organisms in many occasions harbour by pigs in lymph nodes and shed along with feces, urine, saliva and nasal secretions (Lee et al., 2011). Affected pigs manifest the disease as (i) acute septic form, (ii) subacute urticarial form marked by reddish-purple rhomboid spots or "diamonds" in the skin, (iii) joint or arthritic form, and (iv) chronic cardiac form (endocarditis) (Reboli and Farrar, 1989).Various predisposing factors, change of environmental conditions and parasitic infestation lead to

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reappearance of swine erysipelothrix (SE) infection in that population. Seasonal outbreaks of swine erysipelas were investigated in Phek district of Nagaland, India during 2013-2015.

## MATERIALS AND METHODS

### Outbreaks of Swine erysipelas – samples collection

The disease of swine erysipelas was reported from Porba village (altitude 1985 MSL), District Phek of Nagaland during the summer rainy season from 2013 to 2015. The village had a pig population of 552 cross bred and 200 local doom pigs as per the livestock census report 2012 (GOI, 2012). Farmers keep pigs as a back yard small unit mainly for meat purpose and fed them on household as well as hotel waste. In every rainy summer season (May – July) there was disease outbreaks in pigs. Affected animals (150) clinically were anorexic and recumbent with high fever (106°F) for 2-3 days. Some animals developed erythematous patches. Postmortem examination revealed haemorrhages in intestine, congestion of liver, spleen and kidney. During the course of investigation 3 more animals died within 12 days. All affected pigs were also reported to be infested with pig louse *Haematopinus suis*. Clinical sample like swabs from the wound and tissue biopsy from the affected areas were collected in sterile containers for isolation of the causative organism. Samples also included sloughed off tissues and biopsy samples preserved in 10% formalin for histopathological study.

### Virological investigation

Tissue samples were processed for demonstration of classical swine fever using single step Reverse transcription polymerase chain reaction (RT-PCR) (Hoffmann et al., 2005) and swine pox as per the method of Medaglia et al. (2011).

### Identification and antibiotic activity of *Erysipelothrix rhusiopathiae*

The swab samples (N=51) from the affected areas were inoculated into nutrient broth and incubated aerobically at 37°C for 48 h. Sub-culturing was done on blood agar plates, incubated at 37°C in presence of 5% $CO_2$ for 24 h. Colony morphologies were studied. Gram's staining and biochemical analysis were done to confirm the organism. A panel of antibiotic discs containing amikacin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cloxacillin (30 µg), co-trimoxazole (25 µg), enrofloxacin (5 µg), gentamicin (10 µg), neomycin (30 µg), norfloxacin (10 µg), oxytetracycline (30 µg), streptomycin (25 µg) and tetracycline (30 µg) was used to study the antibiotic sensitivity pattern of the isolates. The zone of inhibition was measured, recorded and interpreted according to the clinical and Laboratory Standards Institute criteria (CLIS-MIC).

### Detection of nucleic acid of *Erysipelothrix rhusiopathiae* and sequencing

For detection of *E. rhusiopathiae* nucleic acid tissue samples were processed using DNA Sure® Tissue Mini Kit (Nucleo-pore, cat.#NP-61305) and polymerase chain reaction (PCR) amplification for 16S rRNA gene primers (MO101 F AGATGCCATAGAAACTGGTA and M0102 R CTGTATCCGCCATAACTA) of *E. rhusiopathiae* (Makino et al., 1994) was used. The PCR conditions were optimized with a final volume of 25 µl at 94°C for 5 min followed by 30 cycle of 94°C for 30 s, 54°C for 2 min and 72°C for 45 s and final extension was carried out at 72°C for 5 min. In PCR reaction 28 ng/ µl total genomic DNA was taken along with positive and negative control. Products of PCR were visualized in 2% agarose gel electrophoresis under Geldoc (Kodak, USA). Further PCR products were purified by QIAquick PCR purification kit protocol and sequenced.

### Analysis of gene sequence and phylogenetic studies on *Erysipelothrix rhusiopathiae*

To determine the relationship of the Nagaland isolates of *E. rhusiopathiae* with other isolates of this species, 16S rRNA gene was amplified and sequenced (GenBank accession number KT160358 and KT160359). Sequence identity at nucleotide level was determined by Clustal W method of Meg-Align program in DNASTAR package (DNASTAR Inc., USA). The phylogenetic tree was constructed using other 16S rRNA gene sequences available at NCBI (viz. strain ZYL(KF811052.1), Isolate EU188793.1, strain:KG-BB1(AB055909.1), strain Fujisawa(NR_074878.1), Isolate DQ462571.1, strain: ATCC 19414(AB055905.1), strain JPB251209S(HM569359.1), Chiba9393(EF494748.1), sp. T127_5(JQ739693.1), sp. LV19(KJ670316.1), strain Er.GXLC-1(KP063151.1), inopinata strain 143-02 (inopinata strain 143-02) strain: JCM 8534(LC019778.1) and strain Er.GXBY-1(KP063149.1). Sequences were aligned by ClustalX version 2.1 (www.clustal.org), and the concatenated alignments were used for phylogeny inference (MEGA5; www.megasoftware.net) opting for the Maximum parsimony and Poisson correction. Computed replicates for bootstrap support was done and values were observed.

### Histopathology

Formalin fixed tissues were processed as per standard protocol for histopathological studies. Sections of 4-5 micron thickness were stained routinely with Haematoxylin and Eosin stain and observed under oil immersion objective of a low power light microscope.

## RESULTS AND DISCUSSION

Tissue samples processed for detection of classical swine fever virus and swine pox virus were confirmed as negative for both viral agents. However, bacteriological investigation demonstrated association of *E. rhusiopathiae* infection in affected (5) as well as in dead (2) pigs. Prevalence of erysipelothrix in many animals, mostly in pigs and birds has been reported throughout the world including from India (Shankar et al., 2009; Arora et al., 2011). But there was a single report on swine erysipelothrix (SE) from Meghalaya (Das et al., 2014). North Eastern Region has the highest pig population of the country. Diverse geographical locations, varied climatic situations and frequent movement of pigs favour for spread of the disease through carrier pigs (Leslie et al., 2015). Present report is a thorough investigation on swine erysipelothrix occurred seasonally at Nagaland, another NE state of India.

### Clinical signs

A total of 150 pigs during 2013-15 exhibited clinically high fever (105-107$^0$F), anorexia, firm faeces, and animals

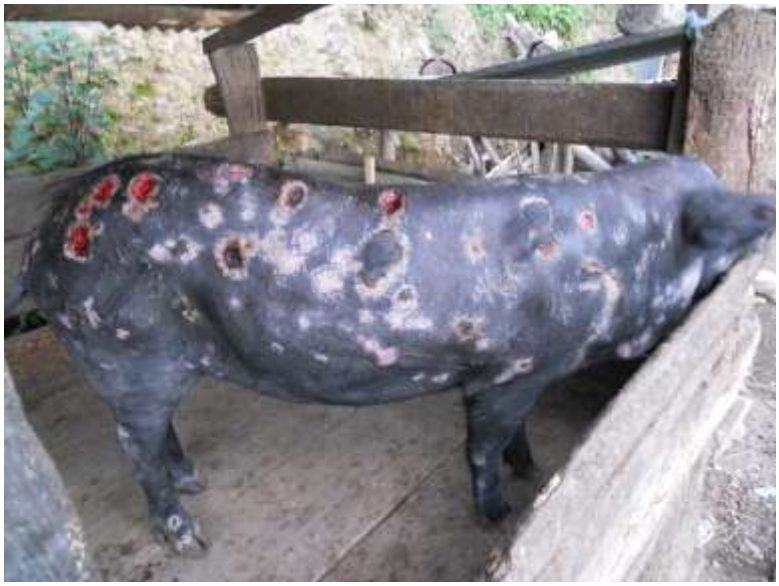

**Figure 1.** Characteristic lesions of sloughed off skin from the cases of *E. rhusiopathiae* infected pigs.

lying down. Cutaneous lesions appeared after 5-7 days of illness initiating with erythematous patches followed by papules of 4-5 mm diameter, dark purple, raised, firm to touch giving square to rhomboid shape in entire body. No vesicular stage was noticed. Although scabs developed in the entire body, prominent lesions were identified on head, face back, belly, limbs, tail and on ear (Figure 1). In unattended cases scab lesions were sloughed off within 17-22 days leaving a large ulcerated area. These typical cutaneous lesions have been mostly seen in grower animals. In affected pigs it appears that cutaneous lesions were developed following acute stages of illness.

Considering the pathogenesis of *E. rhusiopathiae*, organisms gain access to the body, probably through the tonsils or other lymphoid tissue of the digestive tract and spread throughout the body. The bacteria produce neuraminidase, an enzyme that cleaves mucopolysaccharides in cell walls which may mediate the widespread vascular damage that accompanies SE. Vascular damage leads to thrombosis and interference with microcirculation in capillaries and venules at many sites. Classic cutaneous rhomboid urticaria (diamond skin) occurs in a percentage of pigs shortly after the acute febrile stages. In younger pigs with acute erysipelas, signs are similar, with cyanosis of extremities, ears and snouts pronounced and urticaria less common (Anonymous, 2016).

Seasonal occurrence of swine erysipelas in Nagaland justifies the stress due to sudden change of temperature. As stated by Amanda (2012)that stress factors such as overstocking, mixing pigs after weaning, and sudden changes in temperature can trigger clinical erysipelas.

**Isolation and identification of organism**

On blood agar the organism produced small, circular and transparent α- hemolytic colonies with a smooth glistening surface and edge. Biochemically all the isolates were catalase, oxidase and urease negative, produces $H_2S$ and ferments glucose and lactose. All 7 isolates were identified as *E. rhusiopathiae* based on the cultural, morphological and biochemical characteristics. The Nagaland isolates were sensitive *in vitro* to oxytetracycline, tetracycline, ampicillin, amoxicillin and cloxacillin; moderately sensitive to streptomycin, enrofloxacin, amikacin, co-trimoxazole, cefotaxime and ciprofloxacin; and resistant to gentamicin and norfloxacin, chloramphenicol and neomycin. Based on the antibiogram, survived ailing pigs were treated with oxytertacycline at 10 mg/kg body weight, intramuscularly for one week. Eight out of 13 treated animals responded promptly and recovered. Treatment of swine erysipelas cases with penicillin (Shankar et al., 2009) or other penicillin group of drugs such as the combination of amoxycillin and cloxacillin (Das et al., 2014) have been frequently reported.

**Molecular confirmation and characterization**

Tissue samples were negative for Swine fever virus and pox virus in PCR. A total of seven *E. rhusiopathiae* isolates recovered from tissue samples were subjected for PCR amplification using 16s rRNA gene specific primer set. All isolates were found to be positive for *E. rhusiopathiae* with amplification products of 407bp.

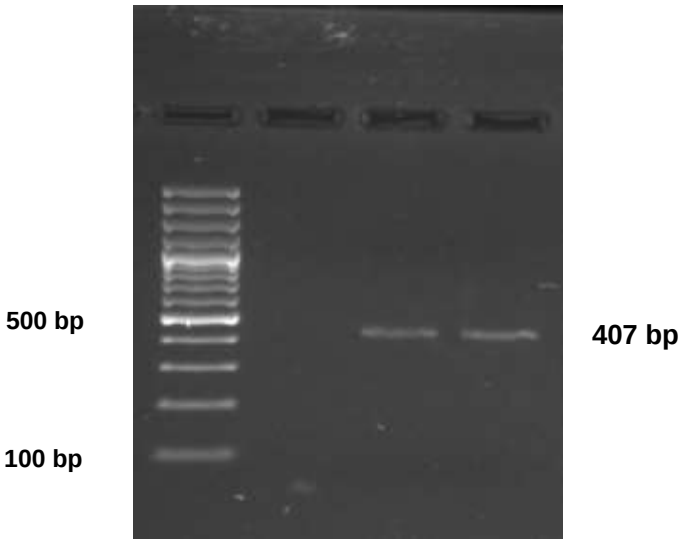


**Figure 2.** 2% agarose gel showing PCR Products. Here, L1-100 bp marker, L2 NTC, L3& 4 PCR products.

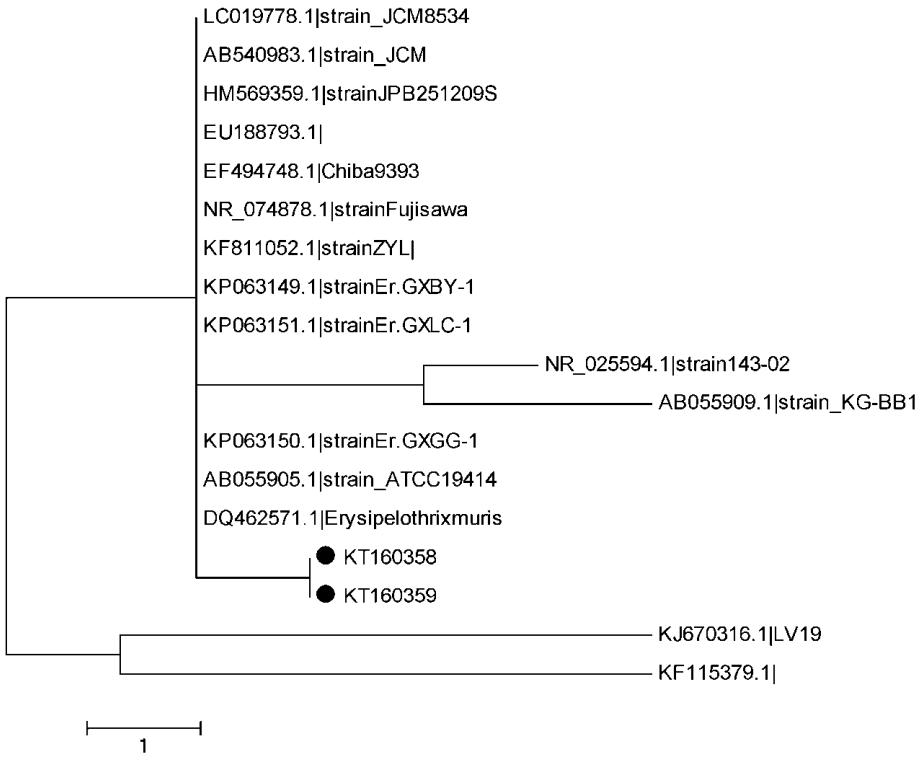


**Figure 3.** Phylogenetic tree of Nagaland isolates of *E. rhusiopathiae* (KT160358, KT160359 as compared to the ATCC 19414 type strain and other isolates.

(Figure 2).

Two Nagaland isolates KT160358 and KT160359 were sequenced and showed high sequence identity with the sequences of other *E. rhusiopathiae* available in the GenBank database. The Nagaland isolates (accession numbers KT160358, KT160359) were in the same clad (Figure 3) along with other strains originated from China. Phylogeny based on the nucleotide and amino acid sequences of the viruses provides a better understanding of the molecular epidemiology of the isolates. The state

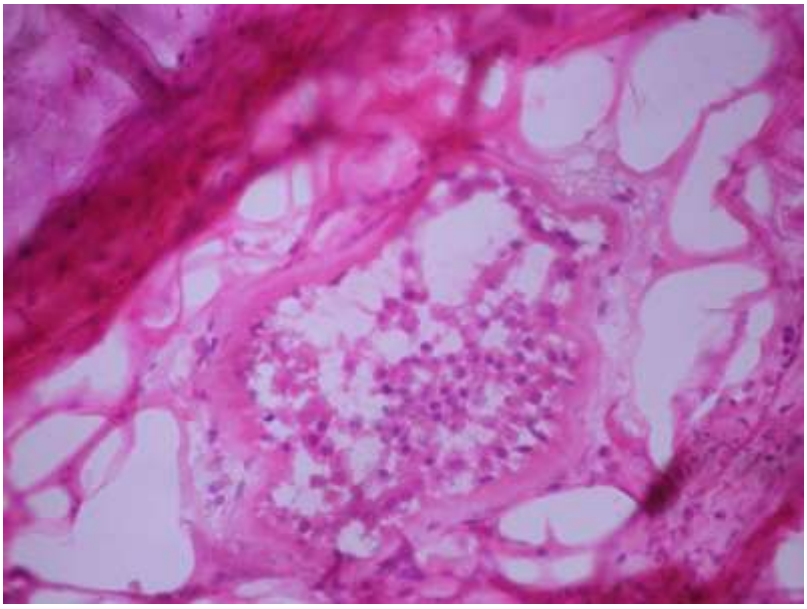

**Figure 4.** Histopathology of skin biopsy sample, H&E, 100X. Congestion and presence of microthrombi in the capillaries of the dermis along with infiltration of lymphoid cells and fibroblast were consistent finding.

Nagaland shares international boundaries with Myanmar and having territorial link with other neighboring international countries like China, Bhutan and Bangladesh. The Chinese strains used in the construction of phylogenetic tree viz. swine isolates KPO 63150.1, strain Er.GXBY-1(KP063149.1), strain Er.GXLC-1(KP063151.1) and murine isolate DQ4625711are forming same clad with Nagaland isolates ( KT160358 and KT160359) indicated an epidemiological link. Movements of animals and animal products might spread the infection to this locality.

## Histopathology

Histopathologic alteration of affected skin showed extensive damage to the capillaries and venules of dermis with infiltration of lymphoid cells and fibroblasts. Congestion and presence of microthrombi in the capillaries were consistent finding in all skin biopsy samples investigated in the present study (Figure 4). Similar observations were also recorded by Shankar et al. (2009).

Cultural characteristics, molecular confirmation and histopathological changes conclusively proved that swine erysipelas is prevailing in Nagaland. Pigs harbouring the infection manifest clinically at sudden change of climate. It is estimated that 30–50% of healthy swine harbour the organism in their tonsils and other lymphoid tissues (Stephenson and Berman, 1978). Again, trans border movement of pigs and their products could facilitate spreading of SE in this locality. Further study on prevalence of swine erysipelas and identification of carrier pigs can provide actual guidelines to control the disease in this part of India.

## Conclusion

Unorganized pig farms in the Phek district of Nagaland experienced high mortality of grower pigs during rainy-summer season. A detail isolation and molecular investigation confirmed the association of swine erysipelas in this part of north eastern region of India. This is the only report on seasonal occurrence of swine erysipelas in the hilly low temperate climate of the NE states of Nagaland, India.

## Conflict of Interests

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Effect of organic and inorganic fertilizer applications on phosphate solubilizing bacteria in the rhizosphere of maize (*Zea mays* L.)

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**A field experiment was conducted between August and October, 2014 at the Department of Microbiology to assess the effect of organic and inorganic fertilizers on the population and activities of Phosphate Solubilizing Bacteria (PSB) in the rhizosphere of maize variety BR9928DMRSR-Y. Three treatment groups were used in the study and these were: Groups which received applications of organic fertilizer (Poultry litter) alone, groups which received applications of inorganic fertilizer (NPK) and the third group which was the control (CON) did not receive any fertilizer application. A total of twenty-three bacteria were isolated and *in-vitro* screening was done for different phosphate solubilization activity. The study revealed that maximum population of total heterotrophic bacteria ($26.8\times10^9$ CFU/g) was obtained with organic fertilizer (OM) treatment. Among the different PSB isolates, OMPSB had the highest bacterial count of $10.2\times10^9$. The lowest bacterial populations were obtained from IFPSB with $6.0\times10^9$ CFU/g. Out of the 23 PSB isolates, 18 were positive for phosphate solubilization with OMPSB8 showing the highest zone with 16 mm. Results showed that application of organic fertilizers enhanced the bacterial population and also showed increase in phosphate solubilization activities in rhizosphere soil compared to NPK and control treatments. This shows that organic fertilizers would be able to sustain the soil fertility for a longer period by meeting the demand of present and future generations.**

**Key words:** Treatment groups, population, phosphate solubilization, *in-vitro* screening.

## INTRODUCTON

Soil nutrient depletion has been a major challenge in Nigeria as a result of continuous cultivation of soils without adequate addition of external inputs. This decline may occur through leaching, soil erosion and crop harvesting (Muchena et al., 2005; Mutegi et al., 2008). Soil nutrients face the risk of continuous decline unless the nutrients are replenished through the use of organic or mineral fertilizers, partially returned through crop residues or through traditional fallow systems. The continued soil nutrient depletion has a negative impact on

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sustainable agriculture as crop production especially that of maize is decreasing (Hussain et al., 2007). Maize (*Zea mays* L.), an annual crop belonging to the grass family Poaceae (OECD, 2003; USDA, 2005), together with rice and wheat are the three most important cereal crops in the world (Min et al., 2015). Maize has the following advantages compared to other cereals: High production, easy processing, easy digestibility, and they are less expensive. It is an important source of carbohydrate, protein, iron, vitamin B and minerals (IITA, 2009) which according to IITA (2001) report, contains 80% carbohydrate, 10% protein, 3.5% fiber and 2% mineral. Thus, it is very essential for food and can also be used as forage and feed for livestock because it is among the highest in net energy content and lowest in protein and fibre content (Oladejo and Adetunji, 2012). In order to maximize the yield of maize crop, improved cultural practices such as organic and inorganic (chemical) fertilizers application can be used.

Though, application of chemical fertilizers can improve the nutrient balance of soils, which may lead to increases in crop yields, its continuous use is hazardous both to human health and the environment (Glick, 2003). This may cause plant toxicity (Nazar et al., 2012) and the bioaccumulation of trace metals in plants may pose a health risk consumed (Khan et al., 2015; Roy and McDonald, 2015). However, the negative effects of chemical fertilizers can be avoided by using organic fertilizers which have a positive effect on the Plant Growth Promoting Rhizobacteria (PGPR) - a group of rhizosphere bacteria found in association with roots which can enhance the growth of plant directly or indirectly by helping plants in nutrient uptake from rhizospheric soil (Mia et al., 2010). Organic fertilizers also play key role in sustaining soil fertility and crop productivity (Soumare et al., 2003; García-Orenes et al., 2016).

Furthermore, to increase the natural fertility of the soil and develop new approaches to reduce the need for chemical fertilizers, PGPR are recognized as important factors in sustainable agricultural production as they may be important for plant nutrition by increasing the P uptake by plants, playing a significant role in the bio-fertilization of crops (Idriss et al., 2007) and serving as a natural source of fertilizers that improve the efficiency of soil and plants (Khalid et al., 2004). The rhizosphere supports large and active microbial population such as bacteria, fungi, nematodes, protozoa, algae and microarthrops (Raaijmakers and Weller, 2001) which play important roles in ecological fitness of their plant host (Kent and Triplett, 2002).

Diverse PGPR strains have been used successfully for crop inoculations and to enhance plant growth (Kumar et al., 2012); these comprise members of the bacterial genera *Azospirillum* (Cassán and García, 2008), *Bacillus* (Jacobsen et al., 2004), *Pseudomonas* (Loper and Gross, 2007), *Rhizobium* (Long, 2001), *Serratia* (De Vleeschauwer and Höfte, 2007), *Stenotrophomonas* (Ryan et al., 2009) and *Streptomyces* (Schrey and Tarkka, 2008). *Pseudomonas* and *Bacillus* genera are the most commonly investigated PGPR, and often the dominating bacterial groups in the rhizosphere (Morgan et al., 2005). The modes of action of PGPR involve complex mechanisms to promote plant growth, development and protection through phytostimulation, bio-fertilization and biocontrol (Bloemberg and Lugtenberg, 2001). Some of these bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus respectively (Hilda and Fraga, 2000; Khiari and Parent, 2005). Phosphate rock minerals are often too insoluble to provide sufficient P for crop uptake; therefore, the use of phosphate solubilizing bacteria (PSB) can increase crop yields up to 70% (Verma, 1993).

The aim of this work was to determine the effect of organic and inorganic fertilizer on the phosphate solubilizing bacteria present in maize plant rhizosphere. In a previous study by Azani (1995), chicken dung showed the best fertilizer compared to cow dung and NPK, thus, chicken dung was chosen as phosphorus source in this study.

## MATERIALS AND METHODS

### Experimental design

The experimental design used for the field experiment was the randomized complete block design as described by Kuntal et al. (2007). The experimental field was sited behind the Department of Microbiology, University of Ibadan, Ibadan, Oyo State. The field was divided into six blocks; two were treated with organic amendments (poultry litter), two with inorganic fertilizer (N:P:K 12:12:17), and two without any treatment and was considered as the control. Grains of maize variety BR9928DMRSR-Y were obtained from IITA, Ibadan headquarters and these were sown by planting three seeds/hole in each block. A total of ten holes were bore in each block.

### Sampling and analysis

Sampling was done for a period of 56 days. Rhizosphere soils were collected at different growth stages of the maize (14, 28, 42 and 56 days after planting), by uprooting four plants from each treatment and keeping the soil around root system intact. After removing the bits of plant roots and other debris, the soil particles which adhered strongly to the roots was immediately used for analysis without drying following the method described by Basul et al. (2010). During the sampling period, population of Total Heterotrophic Bacteria (THB) and Phosphate Solubilizing Bacteria (PSB) in rhizosphere soil was counted at 14 days interval (that is, 14, 28, 42 and 56).

### Isolation and enumeration of rhizosphere bacteria

Three replicate samples of rhizosphere soil were taken for enumeration of total heterotrophic count. The serial dilution plate technique as described by Johnson and Curl (1972) was employed to enumerate the rhizosphere soil by plating on nutrient agar using \

**Table 1.** Physico-chemical properties of soil.

| Parameter | Value |
|---|---|
| pH ($H_2O$) | 7.2 |
| Phosphorus (g/kg) | 8.06 |
| Organic carbon (%) | 38.29 |
| Moisture content (%) | 9.37 |

**Table 2.** Distribution of bacterial isolates from rhizosphere soil.

| Isolates type | Treatments used | No. of isolates | Isolate codes |
|---|---|---|---|
| PSB | Organic manure | 9 | OMPSB1, OMPSB2, OMPSB3, OMPSB4, OMPSB5, OMPSB6, OMPSB7, OMPSB8, OMPSB9 |
| | Inorganic fertilizer | 8 | IFPSB1, IFPSB2, IFPSB3, IFPSB4, IFPSB5, IFPSB6, IFPSB7, IFPSB8 |
| | Control | 6 | CONPSB1, CONPSB2, CONPSB3, CONPSB4, CONPSB5, CONPSB6 |

OM, Organic manure; IF, inorganic fertilizer; CON, control; PSB, phosphate solubilizing bacteria.

the pour plate method. The plates were incubated at 37°C for 24 h. After the incubation period, the colony forming units were counted and expressed as colony forming unit per gram (CFU/g) of soil. Phosphate solubilizing bacteria were isolated by inoculating into sterile Petri dishes of Pikovskaya medium (Pikovskaya, 1948). The plates were incubated at 37°C for 48 h. After the incubation period, the colony forming units were counted and expressed as CFU/g of soil. Colonies with a 'halo' on the different media were sub-cultured onto nutrient agar plates.

The phosphate solubilizing bacteria were isolated using Pikovskaya medium (PVK) because it acts as specific isolation medium for PSB isolation due to the presence of calcium triphosphate which is known for 'halo' zone formation according to Sharma (2005). All the isolates were maintained at 4°C in equal volumes of nutrient broth and 30% glycerol.

#### Determination of soil physico-chemical properties

The following physico-chemical parameters of the soil were determined: pH, organic carbon, moisture content and phosphorus content. pH of the samples was read using an electronic digital pH meter (Table 1). Moisture content was determined by drying the samples in hot air oven at 105°C for 24 h using procedures outlined in the I.I.T.A. manual (IITA, 1979). The total organic carbon was measured by the method given by according to APHA (1985). Available P was determined by molybdenum blue method (Allen et al., 1974).

#### Biochemical tests

All the isolates were characterized by Gram staining and biochemical tests following the methods described by Olutiola et al. (1991). The various tests performed were oxidase, methyl red voges proskauer (MR-VP), indole, citrate, urease, $H_2S$ production and fermentation of various sugars. The morphological and biochemical characterization of the PSB isolates obtained from the rhizosphere of maize was carried out with using 24 h old bacterial cultures. The results were compared with Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).

#### *In-vitro* screening of isolates for phosphate solubilization activity

The isolates were screened for phosphate solubilization ability as described by Gupta et al. (1994). On modified Pikovskaya agar with insoluble tricalcium phosphate, a loop full of each culture was placed on the center of agar plates and incubated at 30°C for 5 days. The solubilization zone was determined by subtracting the diameter of bacterial colony from the diameter of total zone.

## RESULTS AND DISCUSSION

### Distribution of PSB Isolates

A total of 23 isolates were obtained from the soil samples during the 56 days of sampling as shown in Table 2. Twenty-three phosphate solubilizing bacterial isolates designated as PSB were isolated from the different treatments of organic manure (OM), inorganic fertilizer (IF) and non-amended soil which served as control (CON) using Pikovskaya medium. The distribution of these isolates is presented in Table 2. Those isolates considered as PSB were those that formed a 'halo' on Pikovskaya medium. These isolates were subjected to different phosphate solubilization activity screening.

### Total heterotrophic bacteria (THB) counts

The total heterotrophic bacteria (THB) within the rhizosphere of maize plants was counted using Nutrient Agar by pour plate method. THB counts in the rhizosphere of amended soil (organic manure and inorganic fertilizer) and non-amended control soil are presented in Figure 1. The total heterotrophic bacteria population in rhizosphere soil amended with organic

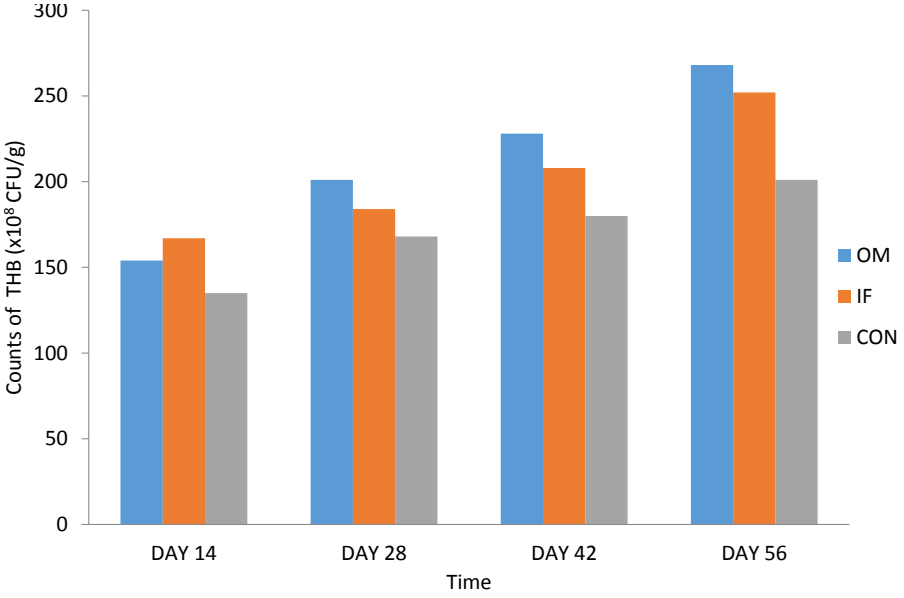


**Figure 1.** Total heterotrophic bacteria count in rhizosphere soil.

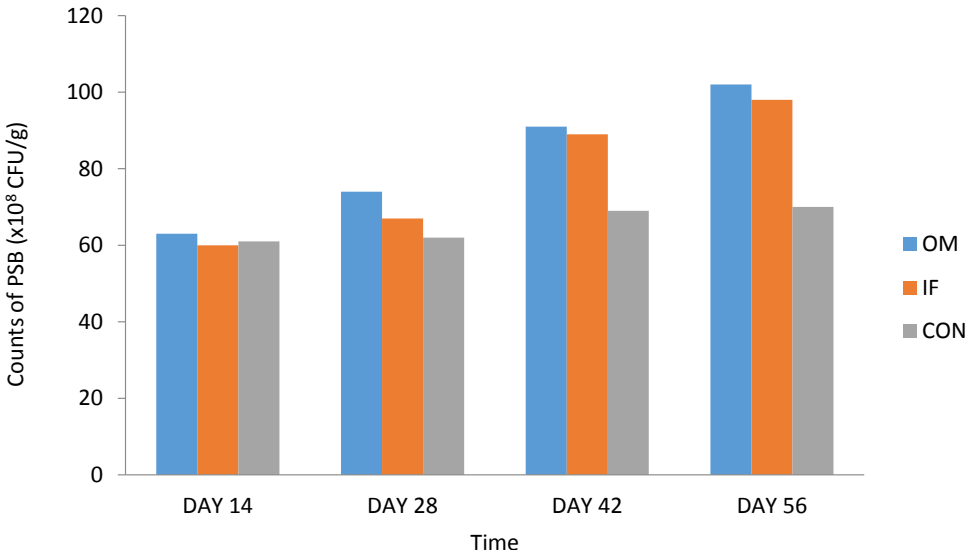


**Figure 2.** The count of phosphate solubilizing bacteria in the rhizosphere of maize.

fertilizer increased from $15.4\times10^9$ CFU/g at day 14 to $26.8\times10^9$ CFU/g at day 56; that amended with inorganic fertilizer increased from $16.7\times10^9$ CFU/g to $25.2\times10^9$ CFU/g while that without amendment (Control) increased from $13.5\times10^9$ CFU/g to $20.1\times10^9$ CFU/g from day 14 to day 56, respectively. All through the period of study, the sample with organic manure treatment (OM) had the highest number of bacteria with $26.8\times10^9$ CFU/g followed by the inorganic fertilizer treatment (IF) having $25.2\times10^9$ CFU/g both at day 56. The inorganic fertilizer treatment had the highest bacterial count of $16.7\times10^9$ CFU/g at day 14. All the additives increased the microbial counts (population) during the period of the study more significantly compared to the control that had a lower microbial population.

## Enumeration of PSB

Phosphate solubilizing bacteria (PSB) within the rhizosphere of maize plant was counted using Pikovskaya medium. The bacteria count of PSB isolates during the period of study is presented in Figure 2. The phosphate solubilizing bacteria in rhizosphere soil amended with organic manure (OM) increased from

**Table 3.** Morphological, biochemical characterization and sugar fermentation of PSB isolates.

| Isolates | Morphology | Gram's Reaction | Catalase | Oxidase | Indole | Citrate | Motility | Methyl Red | Voges Proskauer | $H_2S$ Production | Starch Hydrolysis | Urease | Glucose | Galactose | Sucrose | Fructose | Lactose | Maltose | Mannitol | Probable organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OMPSB1 | R | + | + | - | - | - | - | - | + | - | + | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| OMPSB2 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | + | *Pseudomonas* sp. |
| OMPSB3 | R | - | + | + | - | + | - | - | - | - | - | + | + | + | + | + | - | - | - | *Pseudomonas* sp. |
| OMPSB4 | R | + | + | - | - | - | + | + | + | - | + | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| OMPSB5 | C | + | + | + | - | + | + | - | - | - | + | - | + | - | + | + | - | + | - | *Micrococcus* sp. |
| OMPSB6 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | - | *Pseudomonas* sp. |
| OMPSB7 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | - | *Pseudomonas* sp. |
| OMPSB8 | R | - | + | + | - | + | - | - | - | - | - | + | + | + | + | + | - | - | - | *Pseudomonas* sp. |
| OMPSB9 | R | + | + | - | - | - | - | + | + | - | - | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| IFPSB2 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | - | *Pseudomonas* sp. |
| IFPSB3 | R | + | + | - | - | - | - | - | + | - | + | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| IFPSB4 | C | + | + | + | - | + | + | - | - | - | - | - | + | - | + | + | - | + | - | *Micrococcus* sp. |
| IFPSB5 | R | - | + | + | - | + | + | - | + | - | + | + | + | - | - | + | - | - | - | *Pseudomonas* sp. |
| IFPSB6 | R | - | + | + | - | + | - | - | - | - | + | + | + | - | - | + | - | - | - | *Pseudomonas* sp. |
| IFPSB7 | R | - | + | + | - | + | - | - | - | - | - | + | + | - | - | + | - | - | - | *Pseudomonas* sp. |
| IFPSB8 | R | + | + | - | - | - | - | - | + | - | + | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| CONPSB2 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | + | + | *Pseudomonas* sp. |
| CONPSB3 | R | + | + | - | - | - | - | - | + | - | + | + | + | - | - | + | - | - | + | *Bacillus* sp. |
| CONPSB4 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | - | *Pseudomonas* sp. |
| CONPSB5 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | + | *Pseudomonas* sp. |
| CONPSB6 | R | - | + | + | - | + | + | - | - | - | - | + | + | - | + | + | + | - | - | *Pseudomonas* sp. |

$6.3\times10^9$ CFU/g to $10.2\times10^9$ CFU/g; that amended with inorganic fertilizer (IF) increased from $6.0\times10^9$ CFU/g to $9.8\times10^9$ CFU/g while that without amendment (Control) increased from $6.1\times10^9$ CFU/g to $7.0\times10^9$ CFU/g from day 14 to day 56, respectively. The organic manure treatment had the highest bacterial count with $10.2\times10^9$ CFU/g followed by inorganic fertilizer treatment with $10.2\times10^9$ CFU/g at day 56. Here too, the amended soil treatments had higher growth potential compared to the control soil treatment.

### Identification of PSB isolates

The results of the morphological and biochemical tests are presented in Table 3. The isolates belong to *Pseudomonas* sp. (13), *Bacillus* sp. (6) and *Micrococcus* sp. (2).

### Screening of isolates for PSB activity

A total of twenty three (23) PSB isolates were screened for phosphate solubilization on modified Pikovskaya agar, of which eighteen (18) isolates showed the development of phosphate solubilization zones, ranging from 1.00 to 16.00 mm. Of the 18 isolates that showed zones of solubilization, 6 isolates (OMPSB5, CONPSB4, OMPSB8, IFPSB6, CONPSB5 had CONPSB6) had zones ranging between 6.00 and 16.00 mm while the other 12 isolates showed the development of zones less than 6.00 mm. OMPSB8 and IFPSB6 showed highest phosphate solubilization, that is, 16.00 and 15.00 mm, respectively. The zones of phosphate solubilization of PSB isolates are presented in Figure 3.

Table 4 presents the highest solubilization zones of the isolates from the different treatments during each sampling period (day 14, 28, 42 and 56). For day 14, OMPSB2 had the highest solubilization of 3.00 mm. At day 28, the highest solubilization zone was by OMPSB3 with 5.00 mm. OMPSB5 and CONPSB4 both had highest solubilization zones of 6.00 mm at day 42. However, OMPSB8 and IFPSB6 showed highest phosphate solubilization zone, that is, 16.00 and 15.00 mm respectively at day 56.

Plant growth-promoting rhizobacteria (PGPR) colonize roots of plant and promote plant growth and development through a variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production

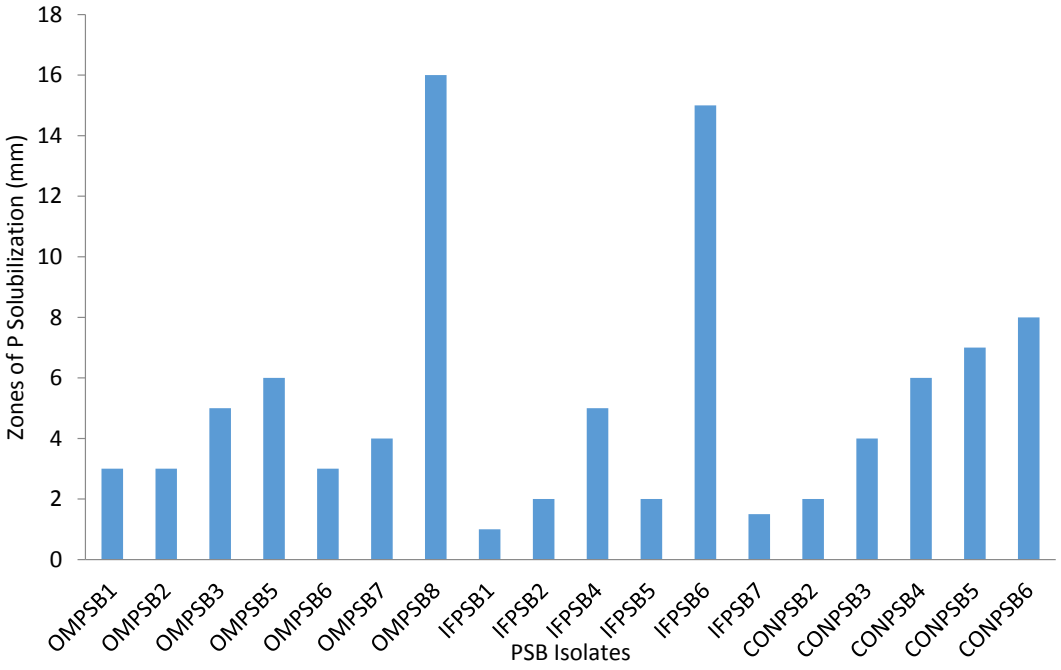


**Figure 3.** The zones of phosphate solubilization of PSB isolates.

**Table 4.** Zones of phosphate solubilization of PSB isolates at different sampling periods.

| Sampling periods | Isolates | Zones of solubilization (mm) |
|---|---|---|
| DAY14 | OMPSB2 | 3.00 |
| | IFPSB1 | 1.00 |
| DAY28 | OMPSB3 | 5.00 |
| | IFPSB2 | 2.00 |
| | CONPSB2 | 2.00 |
| DAY42 | OMPSB5 | 6.00 |
| | IFPSB4 | 5.00 |
| | CONPSB4 | 6.00 |
| DAY56 | OMPSB8 | 16.00 |
| | IFPSB6 | 15.00 |
| | CONPSB6 | 8.00 |

OM, Organic manure; IF, inorganic fertilizer; CON, control; PSB, phosphate solubilizing bacteria.

of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion (Bloemberg and Lugtenberg, 2001).

In the present study, 23 beneficial bacteria were isolated from maize rhizosphere. The bacteria isolates were screened for phosphate solubilization activities and characterized by biochemical tests. Generally, the total heterotrophic counts increased with increase in the duration of maize cultivation just as observed by Liang et al. (2012) in their 15 years study on the effect of fertilizers on soil quality. This can be attributed to the fact that the soil is a suitable medium for the growth of environmental microorganisms because it is rich in nutrients. It has also been reported that the soil receiving manure has larger bacteria pool than in the same soil receiving only chemical fertilizers (Islam and Weil, 2002).

The treatments with organic and inorganic amendments recorded higher bacteria counts as compared to the control treatment. This is similar to the report of Zhao et al. (2014). This observation may be as a result of the

additional nutrients (N, P, K and micronutrients) provided by the additives which the bacteria breakdown for plant to use. This breakdown product in turn leads to the release of more exudates and plant products for use by the rhizosphere bacteria. Hence, increase in rhizosphere bacterial biomass (Das and Dkhar, 2011).

The addition of organic amendments increased the soil bacteria count compared to the inorganic fertilizer and control. Similar observations were made in organic recycling experiments by Chakrabarti et al. (2000) where soil receiving more organic matter tends to harbor higher levels of bacteria with higher microbial activity as proposed by Mäder et al. (2002). The higher bacteria count in OM treated soils is related to the organic matter with respect to decomposition in these materials which is important for proliferation of soil microorganisms in soil.
The bacteria count for the organic manure treatments increased gradually over time and became higher than the inorganic fertilizer application which had a higher bacterial count at day 14. The explanation to this phenomenon can be deduced from the statement of Adegbidi et al. (2003) that the release of nutrients from composts and processed organic manures are generally slower than nutrient release from inorganic fertilizer.

Kumar et al. (2012) isolated and identified *Pseudomonas* spp. as the major PSB in their research. They also reported that *Pseudomonas* spp. are very efficient phosphate solubilizers because they dissolve the soil P through production of low molecular weight organic acids in addition to lowering the pH of rhizosphere. This corroborates the findings in this study where more than 50% of the PSB isolated were identified as *Pseudomonas* sp.

The PSB isolated from the rhizosphere soil with organic manure treatment OMPSB8 (*Pseudomonas* sp.) showed the highest phosphate solubilization zone (16.00 mm) in PVK agar just as a documented by Lazcano et al. (2013). The reports of Zhang et al. (2012) and Yang et al. (2016) have also shown the positive effect of organic manure on the soil microflora. Furthermore, Kohler et al. (2015) showed that organic manure significantly increased shoot biomass by 64%. These reports and the current study show that organic manure application supports/enhances microbial activity in the rhizosphere of plants.

## Conclusion

The application of organic and inorganic treatments significantly affected the rhizosphere bacterial population. Application of fertilizers showed increased bacterial population compared to the control treatment and this is of great importance in nutrient availability in the soil.

Similarly, the plant growth promoting activities of the isolates were also enhanced especially in the treatments with organic manure. The use of organic amendments is efficient, environmentally, cost effective and can be used in place of the more expensive inorganic fertilizer as a viable alternative in the enrichment of nutrient deficient soil. Thus, it can be concluded that the application of organic manure can improve the soil health by improving the bacterial population. This type of study is necessary as it advocates that use of organic fertilizer is an efficient approach to replace chemical fertilizers.

## Conflict of interest

The authors declare that there are no conflicts of interest

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