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# African Journal of Microbiology Research

Table of Content: Volume 8 Number 13, March 26, 2014

## ARTICLES

**Characterization of invertase from *Saccharomyces cerevisiae* MTCC 170**

T. Shankar, P. Thangamathi, R. Rama and T. Sivakumar

**Phylogenetic analysis of genotype VII of new castle disease virus in Indonesia**

NLP Indi Dharmayanti, Risza Hartawan, Dyah Ayu Hewajuli and Risa Indriani

**The associated protein screening and identification in *Proteus mirabilis* swarm colony development**

Yanxia Liu, Jing Xie, Junkang Liu, Yuanxin Deng, Weiwei Xu, Linming Guo and Yu Deng

**Involvement of clathrin and  $\beta$ -arrestins in *Aggregatibacter actinomycetemcomitans* endocytosis of human vascular endothelial cells**

Wang Qin , Li Jie, Yu Jing-Yi, Zhong De-Yu, Ni Jia, Cao Hong and Zhang Jin-Cai

**Bio-efficacy of fungicides, bioagents and plant extracts/ botanicals against *Alternaria carthami*, the causal agent of *Alternaria* blight of Safflower (*Carthamus tinctorius* L.)**

M. R. Taware, V. M. Gholve and Utpal Dey

**Assessment of ants as bacterial vector in houses**

Nara Cristina Silva, Mirian Martins Paiva, Marcos Antônio Pesquero and Lilian Carla Carneiro

**Isolation and identification of probiotic lactic acid bacteria from curd and *in vitro* evaluation of its growth inhibition activities against pathogenic bacteria**

Estifanos Hawaz

**Bacterial diversity in the rumen of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet**

K. C. Das, S. S. Paul, L. Sahoo, K. K. Baruah, P. K. Subudhi, K. Ltu and C. Rajkhowa

**Biofilm in aquaculture production**

P. K. Pandey, Vivekanand Bharti and Kundan Kumar

## **African Journal of Microbiology Research**

**Table of Content: Volume 8 Number 13, March 26, 2014**

**Effectiveness of *Myrothecium roridum* for controlling water hyacinth and species identification based on molecular data**

Orawan Piyaboon, Arm Unartngam and Jintana Unartngam

**Bacterial pathogens associated with infectious diarrhea in King Khalid University Hospital, Riyadh Saudi Arabia 2005-2010**

Somily Ali M, Al-Othman Mohammed F and Kambal Abdelmegeed M

**Detection of selected anaerobic pathogens in primary and secondary endodontic infections in a Turkish population**

Arzu SAHAN KIPALEV, Aysin DUMANI, Fatih KOKSAL, Oguz YOLDAS, Beril AKCIMEN and M. Cem DOGAN

Full Length Research Paper

## Characterization of invertase from *Saccharomyces cerevisiae* MTCC 170

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Activity and stability of invertase obtained from *Saccharomyces cerevisiae* MTCC 170 were characterized with parameters like pH, temperature, metal ions, surfactants and chemical inhibitors. The pH stability of this enzyme was observed between pH 2 and 9 with an average of 63% retaining activity for 24 h. The crude enzyme showed optimum activity at pH 6 and 30°C. Enzyme activity was increased in the presence of 5 mM CaCl<sub>2</sub> (89.11%). Maximum invertase activity of 32.32% was recorded at polyethylene glycol (1%). Maximum invertase activity of 25.58% was recorded at EDTA for *S. cerevisiae* MTCC 170. The kinetic parameters (Km and Vmax) were determined at 30°C and pH 6 for *S. cerevisiae* MTCC 170 invertase for concentrations ranging from 0.5 to 5 mg/ml of sucrose as substrate. The Km and Vmax of *S. cerevisiae* MTCC 170 are 0.6894 mg/ml and 0.3201 µm/min/mg.

**Key words:** *Saccharomyces cerevisiae* MTCC 170, invertase, specific activity, relative activity, enzyme characterization.

### INTRODUCTION

Invertase [ $\beta$ -fructofuranosidases (EC.3.2.1.26)] is the yeast derived enzyme and a member of glycoside hydrolases, which include more than 370 enzymes of plant and microbial origin. Invertase from *Saccharomyces cerevisiae* is the high cost enzyme. Invertases are intracellular as well as extracellular (Ul-Haq and Ali, 2007). Invertase acts on non-reducing fructofuranoside terminal residues of  $\beta$ -fructofuranoside (Veana et al., 2011). The hydrolysis of sucrose which yields an equimolar mixture of glucose and fructose (invert syrup) is sweeter than sucrose due to high degree of sweetness of fructose. Consequently the sugar content can be increased considerably without crystallization of the material. Hence, one of the important applications of

invertase lies in the production of non-crystallizable sugar syrup from sucrose. Due to its hygroscopic nature, invert syrup is used as humectants in the manufacture of soft centered candies and fondants (Gehlawat, 2001).

Invertase is also used whenever sucrose containing substrates are subjected to fermentation viz. production of alcoholic beverages, lactic acid, glycerol, etc. Due to the associated inulinase activity, it is also used for the hydrolysis of inulin (polyfructose) to fructose. Other uses of the enzyme include, manufacture of artificial honey, plasticizing agents used in cosmetics, drug and paper industries and as enzyme electrodes for the detection of sucrose. Enzymatic hydrolysis of sucrose is preferable to acid hydrolysis as it does not result in the formation

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of undesirable flavoring agents as well as coloured impurities (Wiseman, 1975).

Many organisms produce invertase they include, *Neurospora crassa*, *Fusarium oxysporium*, *Phytophthora meganosperma*, *Bacillus macerans* (Kim et al., 2000), *Aspergillus niger*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *Cladosporium cladosporioides* (Almeida et al., 2005), *Arthrobacter globiformis* (Win et al., 2004), *Kluyveromyces marxianus* (Workman and Day, 1983), *Saccharomyces fragilis*, *Aspergillus caespitosus*, *Candida guilliermondii*, *Penicillium chrysogenum*, *S. cerevisiae* (Mona and Mohamed, 2009), *Candida utilis* and *Pichia pastoris* (Ul-Haq and Ali, 2005).

## MATERIALS AND METHODS

### Invertase assay

The culture medium was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used as crude enzyme source for invertase assay. Invertase activity was assayed as per the method of Sumner and Howells (1935) using 0.5 ml of sucrose as the substrate in 0.03 M acetate buffer (pH- 5.0) and incubated at 45°C for 30 min. The reaction was terminated by addition of 1 ml of DNS reagent and tubes were kept at boiling water bath for 5 min. After cooling the tubes at room temperature, 3 ml of distilled water was added in each tube. The intensity of the colour was read at 540 nm in UV-Vis spectrophotometer (Systronics, 119). Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µ mol of glucose/ml/minute under assay condition. Enzyme activity was expressed in International units. Invertase activity was calculated using this formula:

$$\text{IU/ml} = \text{concentration of glucose} / 0.5 \times 30 \times 0.180$$

### Acetone precipitation

For protein precipitation, double the amount of acetone was added to the culture supernatant solution and the solution was left overnight at 4°C, the supernatant was removed and pellet of precipitated protein was kept and dried at laboratory temperature. The pellet which contained invertase was dissolved in 5 ml of double distilled H<sub>2</sub>O and it was dialyzed against double distilled H<sub>2</sub>O for 48 h at 4°C. This was further dialyzed against 50% (W/V) PEG in order to concentrate the protein sample (Mawadza et al., 2000).

### Effect of pH on activity and stability

The optimum pH of the crude invertase was determined by incubating the mixture of the crude invertase in the presence of the buffer (0.1 M citrate buffer) at pH 2 to 12. The pH effects on invertase activity were assayed at pH values ranging from 2 to 12 for 30 min. To determine pH stability, the enzyme was pre-incubated at 30°C for 24 h at pH 2 to 12 (Mase et al., 2008).

### Effect of temperature on invertase activity and stability

The effect of temperature on the activity of invertase was determined in the temperature range of 10-90°C in 0.1 M citrate buffer at pH 6 for 30 min. The invertase activity was determined under standard assay condition. To determine temperature stability,

the invertase was pre-incubated at 30°C for 24 h at pH 2 to 12 (Do et al., 2012).

### Effect of various metal ions on invertase activity

The crude invertase was mixed with 5 mM concentration of various salts such as CaCl<sub>2</sub>, CoCl<sub>2</sub>, MgCl<sub>2</sub>, ZnSO<sub>4</sub>, NiSO<sub>4</sub>, CuSO<sub>4</sub> and KCl for 30 min at 30°C pH 6 before adding the substrate and subsequently invertase activity was determined. To determine metal ions stability, the enzyme was pre-incubated at 30°C for 24 h at pH 6 (Shankar et al., 2010).

### Effect of various surfactants on activity

The effect of surfactants on the activity of crude invertase was determined by pre-incubating the enzyme in the presence of Triton X-100 (1%), Triton X-100 (2%), Triton X-100 (3%), Tween-20 (1%), Tween-20 (2%), Tween-20 (3%), SDS (0.1%), SDS (0.3%), SDS (0.5%), Poly ethylene glycol (0.1%), Poly ethylene glycol (0.3%), Poly ethylene glycol (0.5%) for 30, 60, and 90 min at 30°C before adding the substrate. Subsequently, relative invertase activities were measured at optimum temperature (Patil et al., 2012).

### Effect of different chemical inhibitors on invertase activity and stability

The effect of different chemical inhibitors on invertase activity and stability were determined individually for crude invertase of *S. cerevisiae* MTCC 170. The crude invertase was mixed with 0.1 mM concentration of different chemical inhibitors such as DMSO, EDTA, β-mercaptoethanol, H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> for 30 min at 30°C, pH 6 before adding the substrate and subsequently invertase activity was determined. The relative activities were based on the ratio of the activity obtained at specific chemical inhibitors to the maximum activity obtained and expressed as percentage. To determine chemical inhibitors stability, the enzyme was pre-incubated at 30°C for 24 h at pH 6 (Aziz et al., 2011).

### Determination of kinetic parameters for crude invertase

The kinetic parameters (Michaelis-Menton constant) Km and maximal velocity Vmax of invertase activity of *S. cerevisiae* MK were determined individually from Lineweaver Burk plot optimal assay conditions, 45°C, pH 6 at 30 min for sucrose concentrations ranging from 0.5 to 5 mg/ml. The evaluation of these graph yielded the kinetic parameters for the invertase activity (Graph pad Prism 5.04 software) (Sivakumar et al., 2012).

## RESULTS

### Effect of pH on invertase activity and stability

The effect of pH on the activity of crude invertase was determined in the pH range of 2-12. Maximum invertase activity of 94.28% was recorded at pH 6. The enzyme activity was decreased to 15.14% at pH 12. The pH stability of enzyme was measured by the standard assay method with sucrose. An average 63% of retaining activity was observed between pH 4 and 8 (Figure 1).



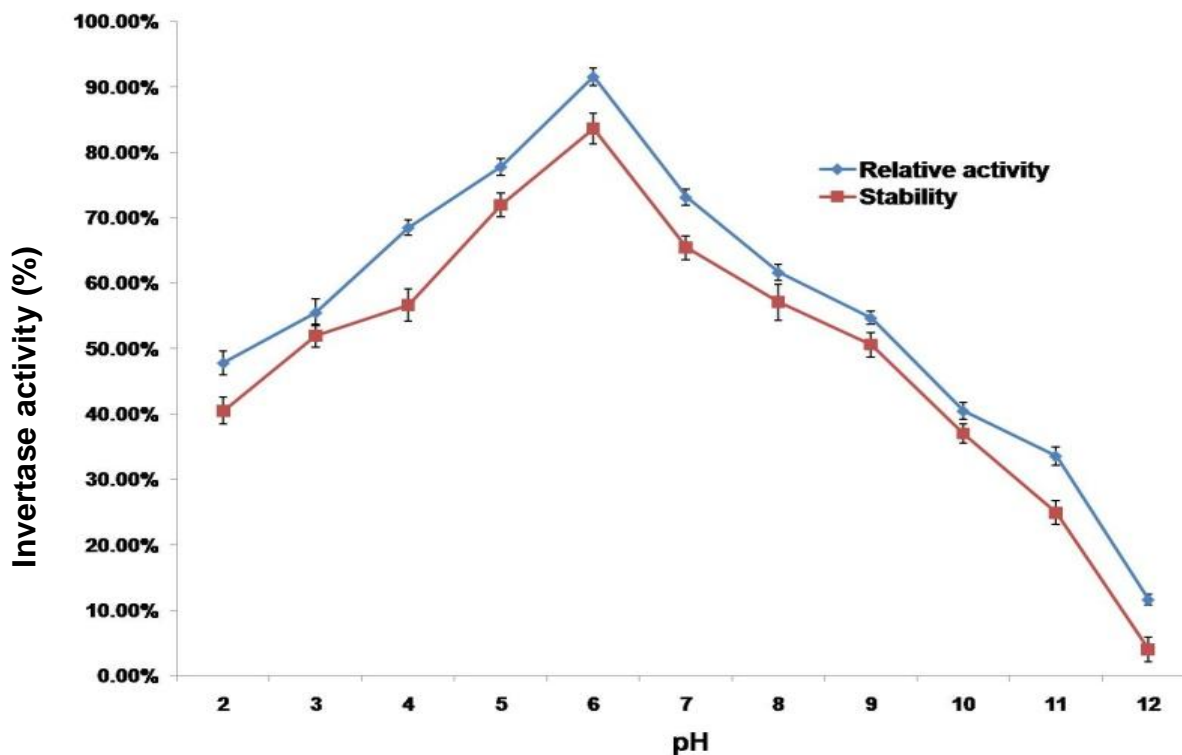


Figure 1. Effect of pH on invertase activity and stability by *S. cerevisiae* MTCC 170.

### Effect of temperature on invertase activity and stability

The effect of temperature on the activity of crude invertase was determined in the temperature range of 10-90°C. Maximum invertase activity of 95.54% was recorded at 30°C. Minimum invertase activity of 21.70% was recorded at 90°C. The original invertase activity was retained from 10 to 90°C approximately above 79% from 10-40°C (Figure 2).

### Effect of various metal ions on invertase activity

The crude invertase was pre-incubated at 30°C for 30 min at different concentration of the metal ions prior to standard invertase activity assay with sucrose. Maximum invertase activity of 89.11% was recorded at calcium chloride. Minimum invertase activity of 11.62% was recorded for potassium chloride. Partial inhibition of the crude invertase was in the order of  $KCl > MnSO_4 > ZnSO_4 > NiSO_4 > MgSO_4 > CoCl_2$  (Figure 3).

### Effect of different surfactants on invertase activity

The relative activity of invertase was decreased with increase in concentration of surfactants and also by time of exposure. At 1% surfactants concentration the relative

activity was high at the same time, at 5% surfactants concentration, it gradually reduced but not completely inhibited. Maximum invertase activity of 32.32% was recorded at polyethylene glycol (1%). Minimum invertase activity of 6.89% was recorded at triton X-100 (1%). The residual invertase activity for surfactants was given in Figure 4a and b.

### Effect of various chemical inhibitors on invertase activity and stability

The crude invertase was pre-incubated at 30°C for 30 min at different concentration of the chemical inhibitors prior to standard invertase activity assay with sucrose. Maximum invertase activity of 25.58% was recorded at EDTA. Minimum invertase activity of 9.30% was recorded at DMSO. The residual invertase activity for chemical inhibitors is given in Figure 5.

### Determination of kinetic parameters for *S. cerevisiae* MTCC 170

The kinetic parameters ( $K_m$  and  $V_{max}$ ) were determined at 30°C and pH 6 for *S. cerevisiae* MTCC 170 for concentrations ranging between 0.5 to 5 mg/ml of sucrose as substrate. The  $K_m$  and  $V_{max}$  of *S. cerevisiae* MTCC 170 are 0.6894 mg/ml and 0.3201  $\mu\text{m}/\text{min}/\text{mg}$

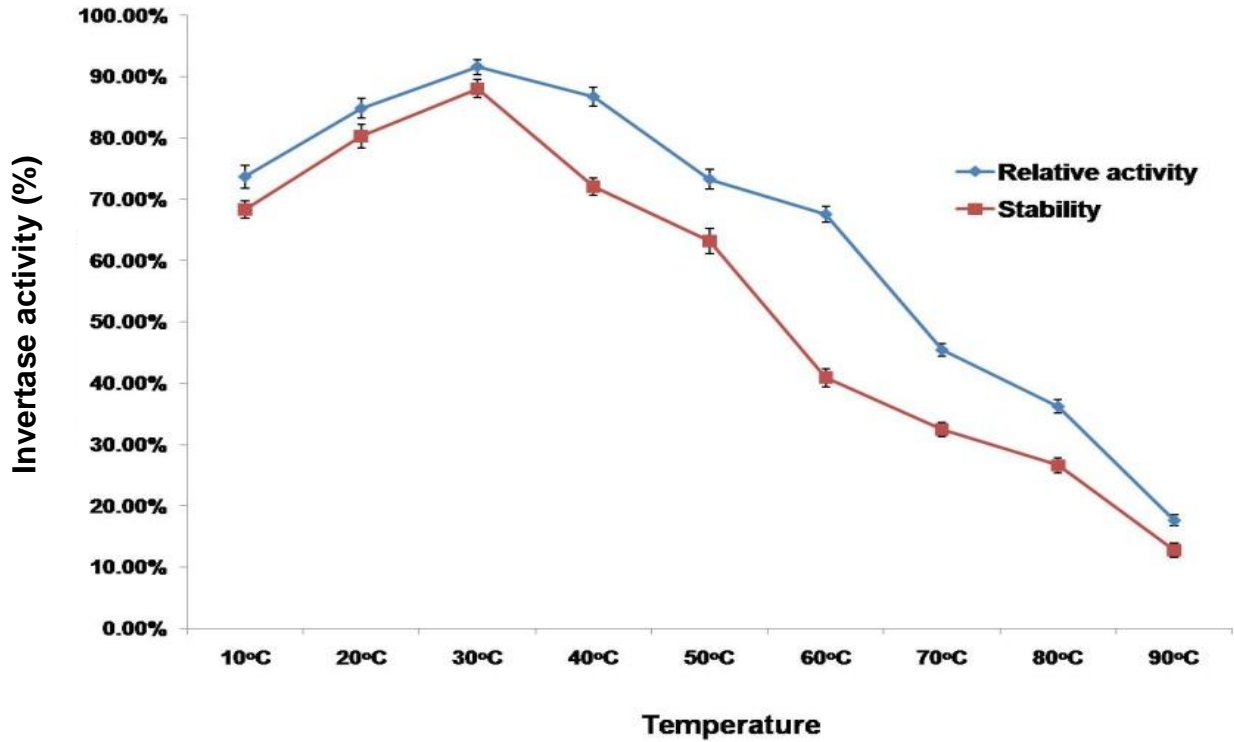


Figure 2. Effect of temperature on invertase activity and stability by *Saccharomyces cerevisiae* MTCC 170.

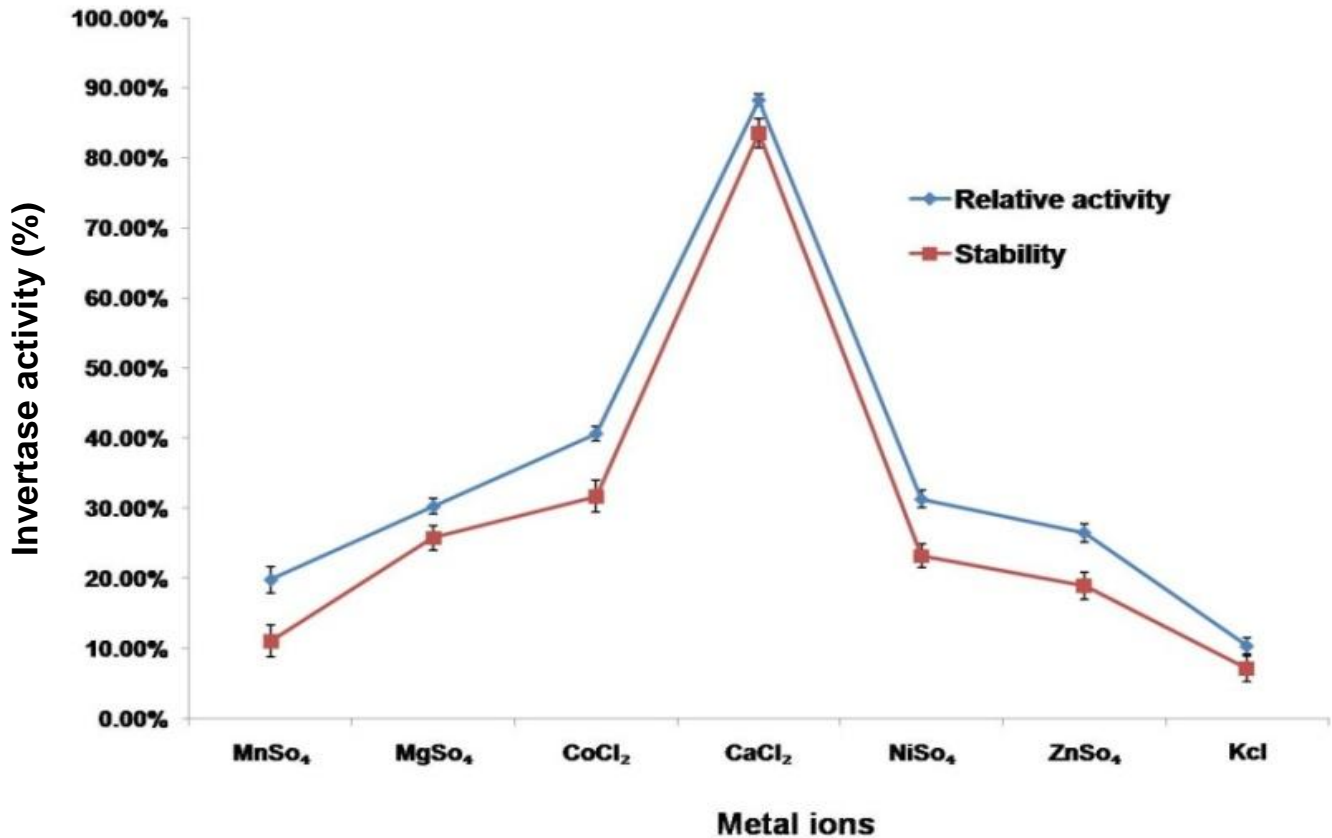


Figure 3. Effect of various metal ions on invertase activity and stability by *S. cerevisiae* MTCC 170.

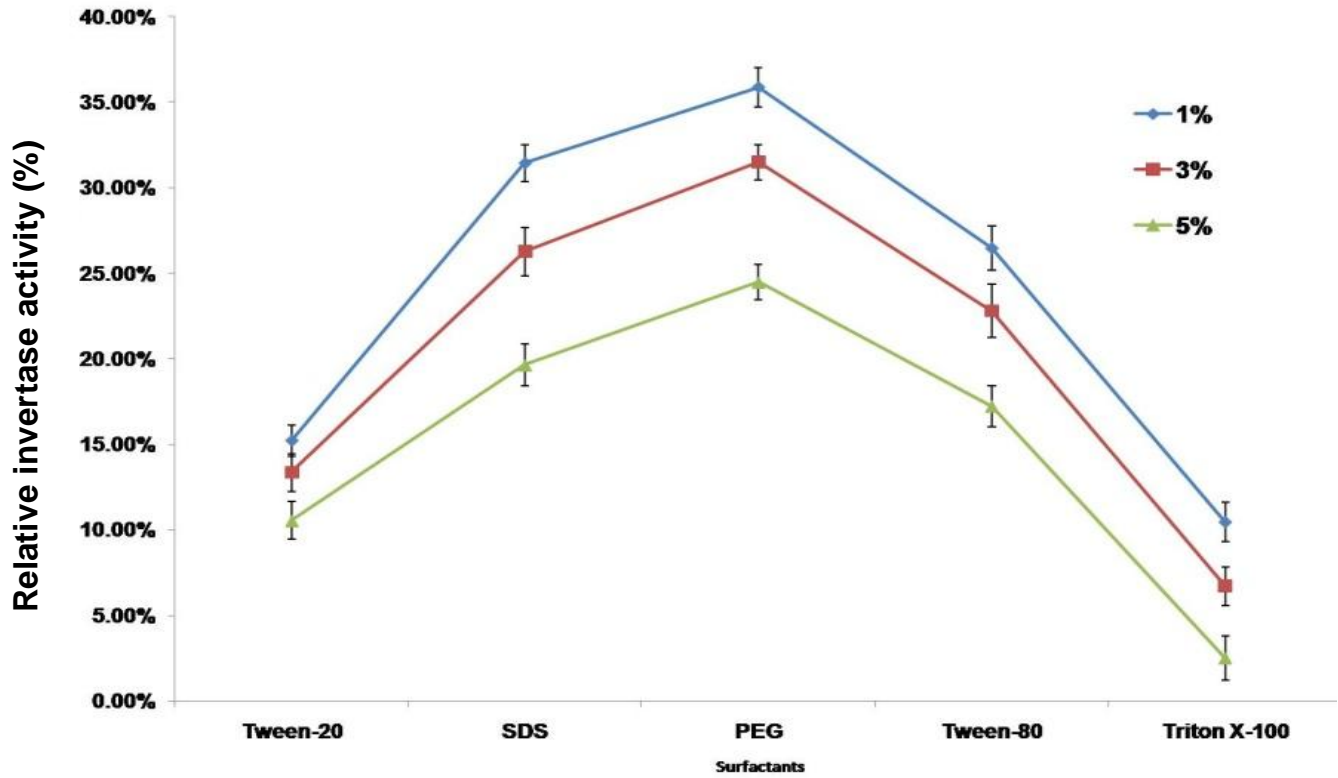


Figure 4a. Effect of different surfactants on invertase activity by *Saccharomyces cerevisiae* MTCC 170.

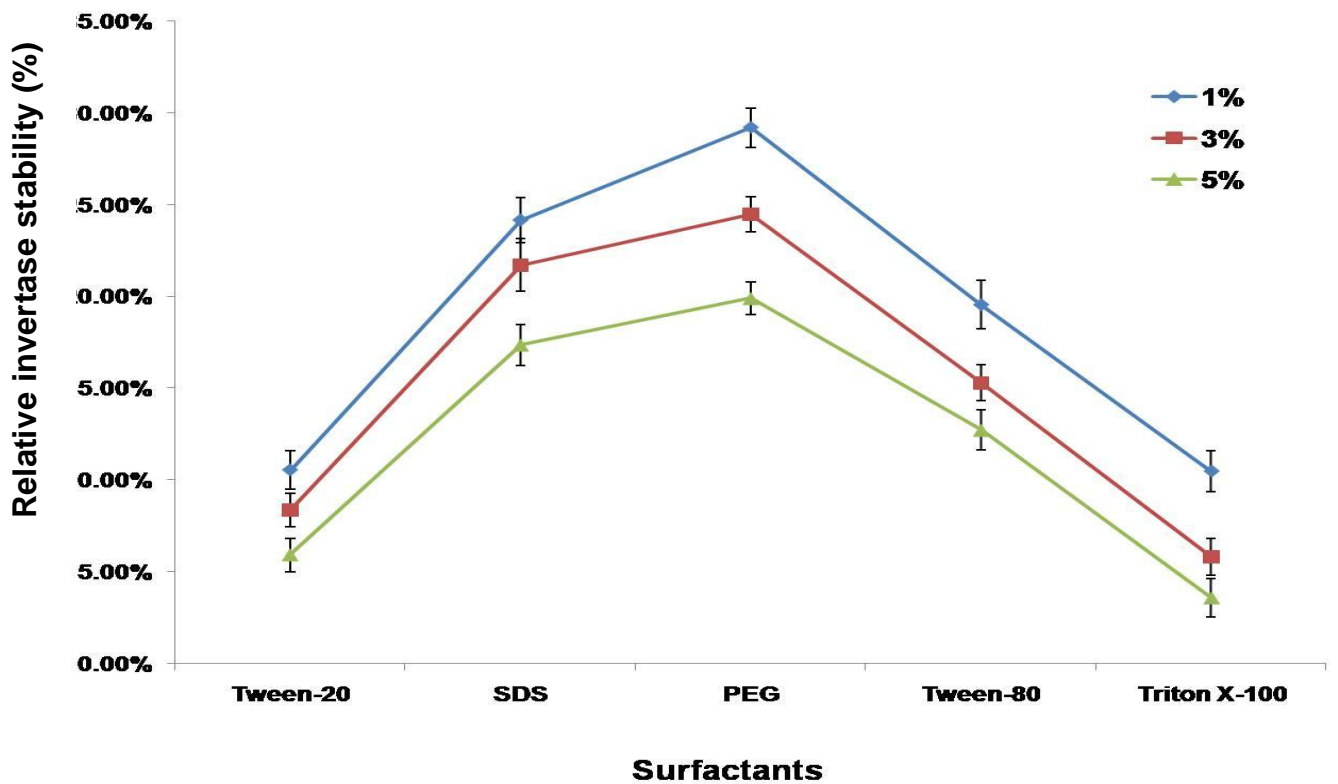
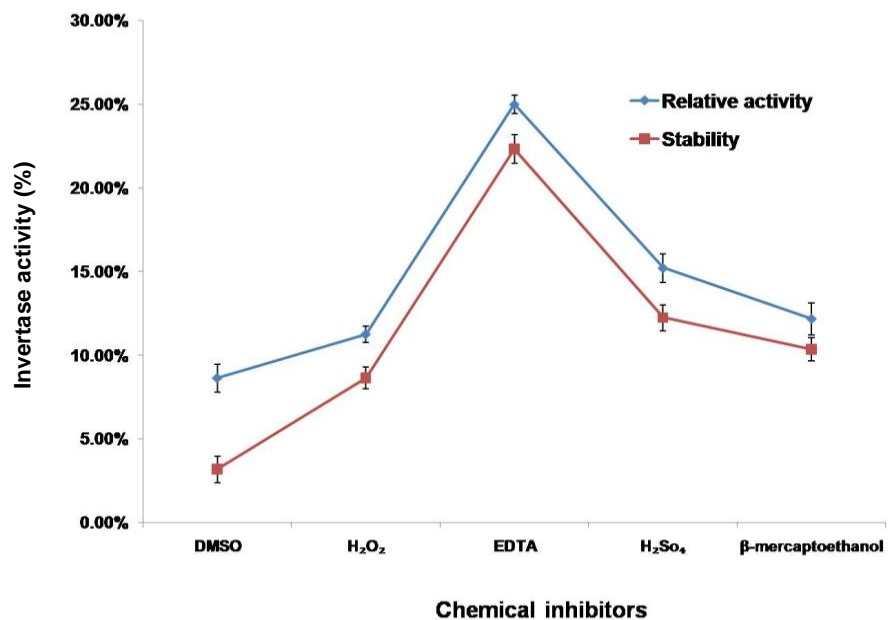


Figure 4b. Effect of different surfactants on invertase stability by *Saccharomyces cerevisiae* MTCC 170.



**Figure 5.** Effect of various chemical inhibitors on invertase activity and stability by *Saccharomyces cerevisiae* MTCC 170.

**Table 1.** Michaelis-Menton constant for *Saccharomyces cerevisiae* MTCC 170.

Michaelis-Menton	<i>Saccharomyces cerevisiae</i> MTCC 170
<b>Best-fit values</b>	
Vmax	0.3201
Km	0.6894
<b>Std. Error</b>	
Vmax	0.05047
Km	0.4680
<b>95% Confidence Intervals</b>	
Vmax	0.2037 to 0.4365
Km	0.0 to 1.769
<b>Goodness of Fit</b>	
Degrees of Freedom	8
R square	0.4760
Absolute Sum of Squares	0.02247
Sy.x	0.05300
<b>Constraints</b>	
Km	Km > 0.0
<b>Number of points</b>	
Analyzed	10

(Table 1). The kinetic parameters of enzymatic reaction were calculated by the Lineweaver-Burk linearization

using the Michaelis-Menton kinetic model, the  $-1/K_m$  value is -1.450 and the  $-r/V_{max}$  is 4.39 (Figure 6).

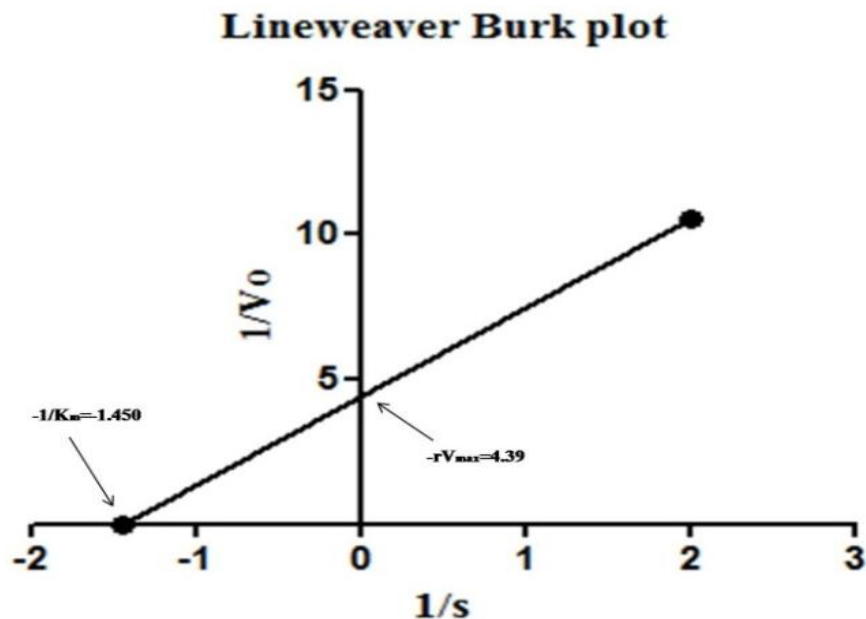


Figure 6. Lineweaver Burk plot for *Saccharomyces cerevisiae* MTCC 170.

## DISCUSSION

In the present work, the effect of pH for invertase activity by *S. cerevisiae* MTCC 170 was assessed. The effect of pH on the activity of crude invertase was determined in the pH range of 2-12. Maximum invertase activity of 91.55% was recorded at pH 6. The invertase activity was decreased to 11.64% at pH 12 in *S. cerevisiae* MTCC 170. Uma et al. (2010) stated that maximum invertase activity was recorded at pH 6.0 for invertase by *Aspergillus flavus*. Patil et al. (2012) evaluated the *Aspergillus* sp. invertase; it gave the good invertase activity for pH 6. Yamamoto et al. (1986) showed that maximum invertase activity was recorded at pH 6.8 for invertase from *Brevibacterium divaircatum*. Invertase exhibits marked stability towards temperature, pH, changes and denaturants. Invertase is used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS) by Uma et al. (2012).

In the present study, the effect of temperature on invertase activity by *S. cerevisiae* MTCC 170 was investigated. The effect of temperature on the activity of crude invertase was determined in the temperature range of 10-90°C. Maximum invertase activity of 91.60% was recorded at 30°C. The invertase activity was decreased to 17.67% at 90°C in *S. cerevisiae* MTCC 170. Whereas, Kaur and Sharma (2005) reported that the 37°C gave good invertase activity for invertase by an actinomycete strain. Similarly, Maria and Rubio (1995) reported that 30°C gave good invertase activity for invertase by *Aspergillus niger*. Whereas, Gine et al. (2010) stated that the maximum invertase activity was recorded at 37°C for invertase by *Lactobacillus reuteri* CRL 100.

The effect of metal ions on invertase activity by *S. cerevisiae* MTCC 170 was investigated in the present work. The various metal ions such as  $\text{CaCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{ZnSO}_4$ ,  $\text{NiSO}_4$ ,  $\text{CuSO}_4$  and  $\text{KCl}$  were tested. Maximum invertase activity of 88.30% was recorded in calcium chloride. The minimum invertase activity was recorded to be 10.34% at potassium chloride for *S. cerevisiae* MTCC 170 invertase. The effect of divalent metal ions  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$  on the activity of the enzyme invertase showed that these ions affected the activity by a certain factor (Shankar et al., 2010). Similarly, Uma et al. (2010) evaluated that the maximum invertase activity was recorded at calcium chloride for invertase by *Aspergillus flavus*. In other hands, Guimaraes et al. (2007) stated that maximum invertase activity was recorded at magnesium chloride for invertase by *Aspergillus ochraceus*. Whereas, Uma et al. (2010) reported that the maximum invertase activity was recorded for sodium chloride and calcium chloride for invertase by *C. cladosporioides*. Moreno et al. (1979) showed that maximum invertase activity was recorded at cobaltous chloride for invertase by *S. cerevisiae* NRRL -Y 12623.

In the present work, the effect of surfactants on invertase activity by *S. cerevisiae* MTCC 170 was investigated. Various metal ions such as Triton X-100, Tween-20, SDS, polyethyleneglycol and Tween-80 were determined. Maximum invertase activity of 32.32% was recorded at poly ethylene glycol. The minimum invertase activity of 6.89% was recorded for Triton X-100 in *S. cerevisiae* MTCC 170 invertase.

The effect of chemical inhibitors on invertase activity by *S. cerevisiae* MTCC 170 was investigated in the present

study. The different chemical inhibitors such as DMSO, H<sub>2</sub>O<sub>2</sub>, EDTA, H<sub>2</sub>SO<sub>4</sub> and β-mercaptoethanol were tested. Maximum invertase activity of 25.58% was recorded in EDTA. The minimum invertase activity was recorded at 9.30% of DMSO in *S. cerevisiae* MTCC 170. Uma et al. (2012) reported that the maximum invertase inhibitory activity was recorded in toluidine for invertase by *C. cladosporioides*.

In the present investigation, the kinetic parameters (Km and Vmax) were determined at 30°C and pH 6 for *S. cerevisiae* MTCC 170 for concentrations ranging between 0.5 and 5 mg/ml of sucrose as substrate. The Km and Vmax of *S. cerevisiae* MTCC 170 are 0.6894 mg/ml and 0.3201 μmol/min/mg. Hocine et al. (2000) reported similar result for invertases from *A. niger*, Km and Vmax values for each enzyme were determined using the Lineweaver–Burk plots which were calculated to be 44.38 mM and 1030 mmol ml<sup>-1</sup> min<sup>-1</sup> for FTS and 35.67 mM and 398 mmol ml<sup>-1</sup> min<sup>-1</sup>. Ghazi et al. (2000) investigated the behaviour of fructosyltransferase from *A. aculeatus*, determined the kinetic constants (Km and Kcat) for both hydrolysis and transfer reactions. The reaction rates (μmol mg<sup>-1</sup> min<sup>-1</sup>) of sucrose concentrations up to 1.75 M were plotted.

Chang et al. (1994) reported that purified enzyme had an optimal pH (5-6), temperature (50°C) and a Km value of 0.53 M for catalyzing self transfer reaction from sucrose. Gine et al. (2010) reported that for invertase in *Lactobacillus reuteri* (CRL 1100), the Km (6.66 mM) and Vmax (0.028 μmol/min) values for sucrose were obtained. Workman and Day (1983) reported the Km value for sucrose was 13.6 mM in *Kluyveromyces fragilis*. Similar findings with the Bhatti et al. (2006) obtained Km value of 3.57 mM for sucrose in *Fusarium solani*. Hernalsteens and Maugeri (2008) reported the Km (13.4 g/l) and Vmax (21 μmol/ml/min) for sucrose by invertase in *Candida* sp. Buttner et al. (1990) determined Km value (71-83 mM) for sucrose in *Trichosporon adeninovorans* for two internal invertases. Whereas, Aziz et al. (2011) showed that the kinetic parameters of IM was about 1.74-fold more active (p=0.0002) than IW because their Vmax values were 564 and 325 U/mg of protein/min.

## Conclusion

The yeast (*S. cerevisiae* MTCC 170) capable of producing invertase was obtained from MTCC. The invertase activity and stability for the *S. cerevisiae* MTCC 170 was found to be at pH 6, 30°C, calcium chloride (metal ions), poly ethylene glycol (surfactants), EDTA (chemical inhibitors). The Km and Vmax of *S. cerevisiae* MTCC 170 are 0.6894 mg/ml and 0.3201 μmol/min/mg.

## Conflict of interests

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

## ACKNOWLEDGEMENT

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Full Length Research Paper

## Phylogenetic analysis of genotype VII of new castle disease virus in Indonesia

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Newcastle disease (ND) is a very contagious disease in chickens and turkeys and one of the most important diseases of poultry in the world. The infection causes sudden death with high mortality. In Indonesia, Newcastle disease in recent years showed symptoms slightly different from previous symptoms of this disease. NDV infection of genotype VII has been reported to cause this outbreak in several commercial poultry farms in Indonesia. This study aimed to isolate and identify the recent ND virus and determine group of genotype of ND in Indonesia for the development of seed of ND vaccines expected to be more effective in the control of Newcastle disease in the field. The method used in this study includes the collection of samples from the field, ND virus isolation, RT-PCR and DNA sequencing of the F and HN genes of ND viruses. Our result showed that six isolates belong to genotype VII of ND viruses, one isolate belong to genotype VI and the other isolate belong to genotype I. The prediction of pathotypes of amino acids sequence on F gene of NDV indicated that seven isolates have motif R-R-R-K-R and R-R-Q-K-R which is a marker for pathotype for velogenic of ND viruses. The cleavage site of amino acid sequences from one isolate (RIVS isolate) has G-K-Q-G-R-L which is lentogenic pathotype of NDV. This study indicated that genotype VII viruses were predominant virus circulating in the field and we suggested the update of master seed vaccine of ND in Indonesia.

**Key words:** New castle disease (ND), genotype VII, phylogenetic analysis.

### INTRODUCTION

As an OIE list A categorized disease, the outbreak of Newcastle disease (ND) has severely affected poultry industries world-wide causing massive economic repercussion. The distribution of this highly contagious and infectious disease has already widely spread across many regions of the world. The etiological agent belongs to virulent serotypes of avian paramyxovirus type 1 (APV-1) of the genus *Avulavirus*, subfamily *Paramyxovirinae* and family *Paramyxoviridae* (ICTV, 2005). At least ten

serotypes of avian paramyxovirus (APV1-APV10) have been recognized up to date (Miller et al., 2010). The genome of this enveloped virus is nonsegmented, single-stranded, negative sense RNA with approximately 15 kilo base pair (kbp) for entire genome size. The genome encodes for at least six major proteins including nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large polymerase (L) (Krishnamurthy and Samal, 1998; De Leeuw and

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Peeters, 1999). Two non-structural proteins (V and W) are expressed at transcription process of P gene (Peeters et al., 2004; Steward et al., 2003). Moreover, the HN and F proteins are acknowledged to play important role in the virulence characteristic (Huang et al., 2004). These two surface protein surfaces are involved in the attachment and membrane fusion in the initial infection on the host cells (de Leeuw and Peeters, 1999; Lamb and Kolakofsk, 1996).

Manifestation of the disease may vary from subclinical to severe or systemic infection with high mortality rate depending on the virulence of virus strain and the host state and susceptibility. Based on the clinical symptoms in chicken, the NDV are grouped into five pathotypes including viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic respiratory and asymptomatic enteric (Alexander and Senne, 2008). The concern about ND infection in the poultry industry should not be oriented only to the pathogenic strains since the apathogenic strains may contribute to economic drawback as well. The avirulent strain of APV1 could result in decreased productivity of the infected farms such as drop of egg production or poor body weight performance (Aldous and Alexander, 2003; Leuck et al., 2004; Ojok and Brown, 1996). Farmer's ignorant on the significance of avirulent ND strain in poultry farms may lead to unexpected income loss.

Historically, the outbreak of ND infection in Indonesia has been isolated more than eighty years ago (Kranefeld, 1926). Some Diseases Investigation Center in Indonesia reported the isolation of NDV from outbreak occurring in their areas. The ND infection has become endemic in most regions of the country causing significant economic losses even in Indonesia. However, the ND outbreaks are properly controlled by implementing intensive vaccination program in the commercial poultry farms. Numerous type of vaccines are available from several commercial sources including live and killed vaccine which consist of the Lasota strain for either single application or combination with other poultry diseases vaccines. Despite the fact that ND infection is overshadowed by current Asian highly pathogenic avian influenza H5N1 outbreak even in Indonesia, the ND viruses still pose threat to the poultry industries by maintaining their evolution and spread in the farm environment. In Indonesia, failure of the protection of the current vaccination program against ND infection perhaps caused by the seed vaccine did not match with the field NDV, mutation, or introduction exotic strain. Lately, NDV infection of genotype VII has been reported to cause disease outbreak in several commercial poultry farms in Indonesia. The objectives of this study were to characterized recently isolated ND viruses by molecular approach as well as to determine genotypic grouping of ND viruses in Indonesia in order to look for candidate of new master seed vaccine that more effectively control ND outbreak in the field.

## MATERIALS AND METHODS

### Samples collection and screening for ND virus

Field samplings were conducted in several commercial chicken farm in Sukabumi (West Java Province) and Tangerang (Banten Province). Both districts have experienced recurrent outbreak of ND because of high density of poultry population in these areas. Type of sample collected in the fieldwork is chicken sera and cloacal swab. A sterile cotton-tipped swab was used for sampling of cloacal swab. Subsequently, the swabs were stored in viral transport medium consisting of Dulbecco's modified eagle medium (DMEM) with addition of antibiotic and antifungi. The samples were immediately transported to the Virologi Laboratory, IRCVS, Bogor for further analyses. The presence of antibody against ND was examined in the serum samples by hemagglutination inhibition (HI) test using OIE standard methodology.

### Isolation ND viruses

Virus isolation was conducted by inoculating cloacal samples into specific pathogen free (SPF) of embryonated chicken eggs (9-11 days) only for ND positive sample by RT-PCR test. Briefly about 1000 µl sample in transport medium was homogenized by vortexing and subsequently centrifuged with the speed of 2500-3000 rpm. Then, supernatant was inoculated into embryonated egg via allantoic cavity with 4 days observation for death of embryo. Subsequently, the allantoic fluid was harvested and screened for the virus presence by rapid agglutination test for 10% chicken red blood cells in the porcelain plate. The positive agglutinated allantoic fluid was retested again by RT-PCR for the confirmation of ND viruses. Furthermore, the ND virus isolates were kept in the freezer at -20°C for further analyses.

### Sequencing of PCR products

Molecular characterization of ND virus isolates were accomplished by sequencing the F and HN gene. Briefly, RNA of ND virus was isolated using Viral RNA minikit (Qiagen). Subsequently, the RT-PCR test designed by Liu et al. (2008) was performed to amplify the F gene with size of product in about 535 base pairs (bp). Meanwhile, the amplification of HN gene was carried out using Peroulis-Kouritis et al. (2002) method to generate product with size of 320 bp. The RT-PCR for both genes was conducted using the Superscript III One Step RT-PCR system (Invitrogen).

The DNA products were separated by gel electrophoresis and visualized by UV transilluminator. Furthermore, specific amplification products were purified using QIAquick Gel Purification System (Qiagen) and quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.). The DNA sequencing was performed by BigDye Terminator Cycle v3.1 Cycle Sequencing Kit (Applied Biosystem) in Genetyx Analyzer 3130 machine (Applied Biosystems, USA).

### Phylogenetic analysis and predicted amino acid of cleavage site

The results of nucleotide sequence were verified and edited using Bioedit version 8 (<http://www.mbio.ncsu.edu/BioEdit>). The sequences of the F and HN gene were compiled and compared with the ND nucleotide sequence database in the genbank NCBI. Nucleotide analysis, prediction of amino acid sequences and multiple sequence alignment was done with Clustal W (BioEdit 8) and MEGA 5.2. Phylogenetic tree analyses for genotyping were generated by neighbor-joining bootstrap analysis (1,000 replicates) using the

**Table 1.** Field samples for identification of ND viruses using RT-PCR.

District/province	Total farm	Total samples	Positive by (RT-PCR – F gene)	Virus isolation	Remark
Sukabumi	16	171	22	Negative	No clinical signs
Tangerang	7	73	36	Negative	No clinical signs
East java	1	5	1	Positive	High mortality
West java	1	5	1	Positive	High mortality tinggi

**Table 2.** Historical isolates of ND in Indonesia.

Name of virus	Species	Area
I-337	Chicken	West Java
I-14	Chicken	West Java
I-171	Chicken	East Java
I-237	Chicken	Kupang, NTT
I-53	Chicken	West Java
RIVS	Chicken	No data

Tamura-Nei algorithm in MEGA version 5.2 software (<http://www.megasoftware.net>).

## RESULTS

### Identification of NDV

The samples were collected from commercial layer farms in two districts, that is, in Sukabumi and Tangerang. In addition, several samples were also obtained from broiler farms in East Java Province. Hemagglutination inhibition test revealed that all the poultry farms where vaccination was done showed various value of antibody titers in the range of between 5-11 log<sub>2</sub> (data not shown). Most of chicken farms may have problem with NDV even though vaccination is routinely implemented. The RT-PCR assay identified presence of NDV in numerous cloacal swab samples (Table 1); however, only two isolates viruses of ND can be grown in embryonated eggs of SPF chicken. In this study, we did further analyze DNA sequencing for the new isolated viruses, that is, GTT/11 and SME/13 for as well as six other NDV isolates from our laboratory, namely I-337, I-14, I-171, I-1237, I-53 and RIVS (Table 2). The DNA sequencing was successfully accomplished from the RT-PCR product of F and HN gene in about 535 base and 320 bp, respectively.

### Phylogenetic analysis

The phylogenetic tree analysis of F gene demonstrated that most of ND isolates used in this study belong to genotype VII. The newly isolated NDV, that is, GTT/11 and SME/13 are classified in genotype IV that have close relationship with the other Indonesian GVII viruses isolated in 2005. Despite the fact that the other four historical isolates (I-337, I-14, I-171 and I-1237) also belong to

genotype VII, they showed more similar relationship with the other GVII viruses isolated in 1990 (Figure 1). Moreover, the I-53 and RIVS are categorised as genotypes I and VI, respectively. Concurrently, the phylogenetic tree analysis of HN gene showed that GTT/11 and SME/13 isolates have close genetic proximity with other Indonesian ND viruses isolated in 2010 (Figure 2).

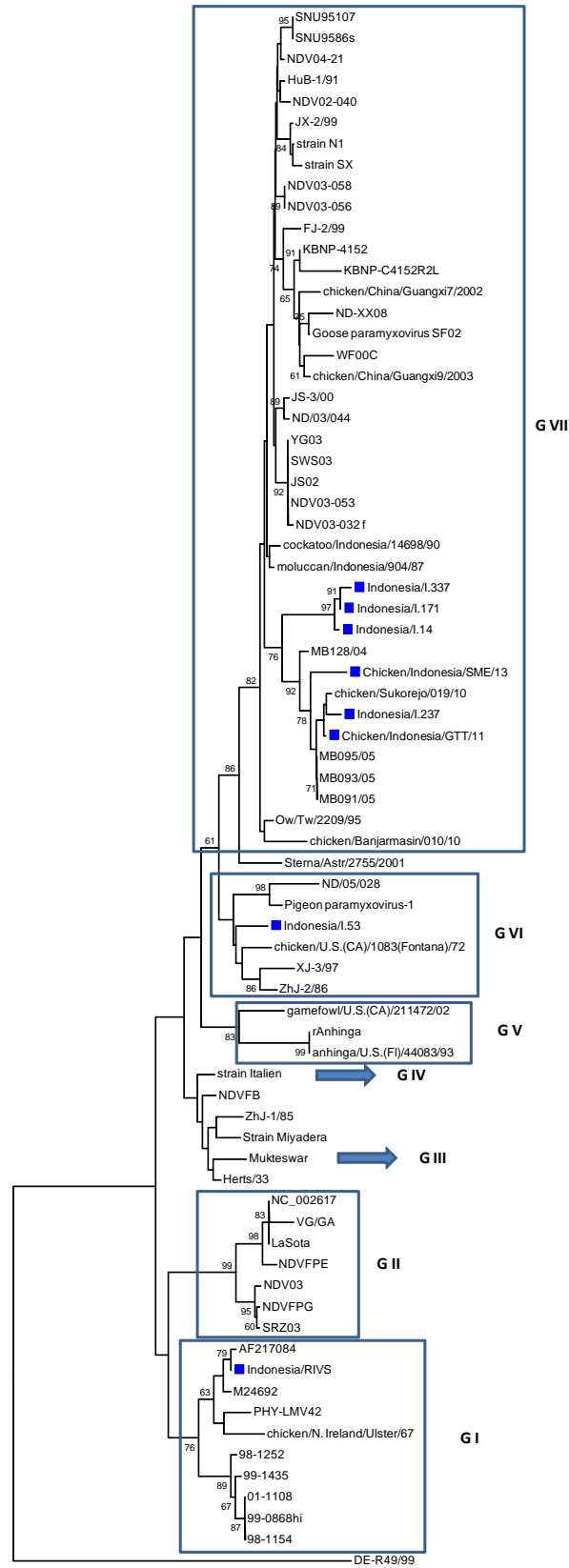
### Proteolytic cleavage site of F0 protein

The genetic analyses of amino acid sequences of the NDV isolates were compared with the NDV database from GenBank (NCBI). Based pathotypes prediction on cleavage site of fusion protein, six isolates (GTT/11, I-337, I-171, I-14, I-237) have motif of amino acid R-R-R-K-R and two other isolates (I-53 and SME/13) exhibit motif of R-R-Q-K-R. These amino acids sequence motifs are marker for velogenic or mesogenic pathotypes. Conversely, the amino acid motif of RIVS isolate is G-K-Q-G-R-L, which is marker for lentogenic pathotype of NDV (Figure 3).

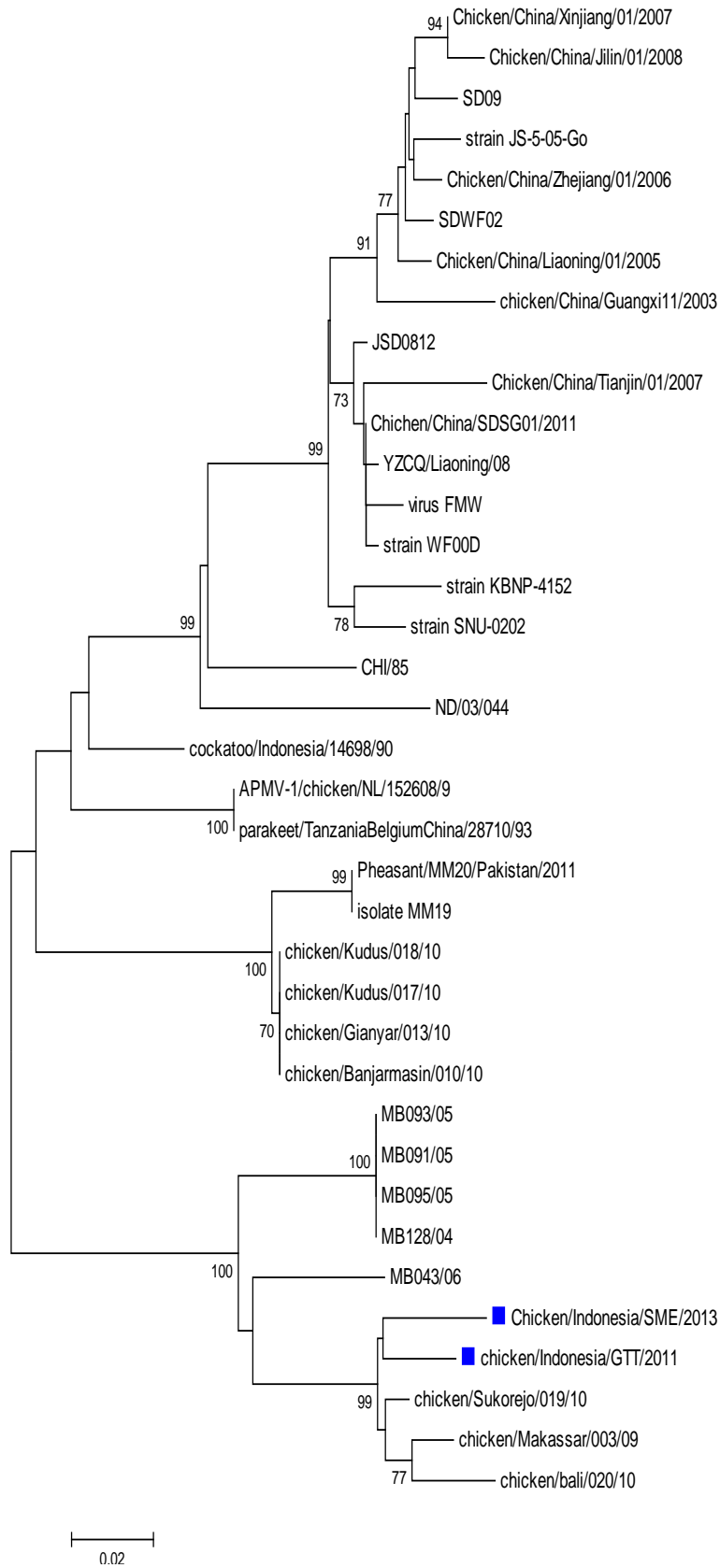
## DISCUSSION

Amino acid sequence of cleavage site on F gene can be used to predicted pathotypes of NDV. This sequence analysis of the F protein can be done instead of conventional methods such as mean death time (MDT) and intracerebral pathogenicity index test (ICPI) (Panda et al., 2009). Pathotype and virulence of NDV can be predicted from amino acid sequence on cleavage site of F0 protein after post-translational modification. The amino acid sequence at the F protein cleavage site is different among most lentogenic, mesogenic and velogenic NDV strains (Millar et al., 1988). Mostly, virulent strains comprise motif <sup>112</sup>R/K-R-Q-K/R-R<sup>116</sup> at the C-terminus of the F2 protein and phenylalanine (F) at residue 117 at N-terminus of the F1 protein. Meanwhile, the low virulent strains retain motif <sup>112</sup>G/E-K/R-Q-G/E-R<sup>116</sup> in the same region and leucine (L) at the same position (Collins et al., 1993; Panda et al., 2004).

In our study, seven isolates have multibasic amino acids sequence on cleavage site that indicated the velogenic and mesogenic NDV strains (Seal et al., 1995). On the other hand, one isolate is classified as lentogenic strain because it has monobasic amino acid motif in



**Figure 1.** Phylogenetic tree of partial of Fussion (F) gene of ND viruses. Indonesian viruses used in this study are shown in blue color. The region of the fussion from 198-450 was analyzed using MEGA version 5.2. A neighbor-joining bootstrap analysis (1.000 replicates) using the Kimura-Nei model.



**Figure 2.** Phylogenetic tree of hemagglutinin-neuraminidase (HN) gene of ND viruses. Viruses used in this study are shown in blue color.

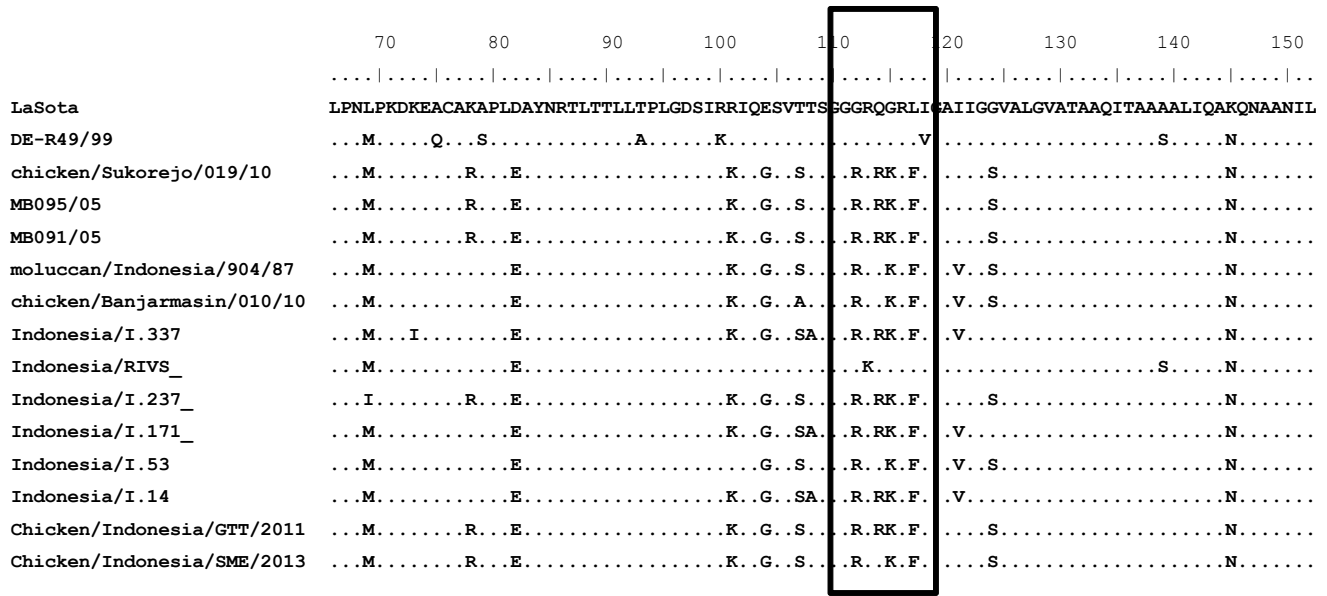


Figure 3. Cleavage site of amino acid sequences prediction on F gene of ND viruses as shown in the black color box.

cleavage site on F protein (Seal et al., 1995).

Based on phylogenetics, ND viruses are classified into two classes, namely Class I and II. Class I of ND viruses have genome sizes typically 15198 nucleotides which were isolated from waterfowl and domestic poultry and usually are avirulent. Class II of ND viruses are a group of viruses most virulent and avirulent viruses with some genotypes I- IV found prior to 1960 with the genome size of 15186, while genotype V-VIII, a new strain that was isolated after 1960 with a genome size of 15192 nt. genotype VII and VIII are new genotypes found after 1980 that caused a pandemic in Europe, the Far East and South Africa (Czegledi et al., 2006; Herczeg et al., 1999; Ke et al., 2001; Lomniczi et al., 1998; Abolnik et al., 2004; Liu et al., 2007). The results of our study showed that the ND virus used in this study was six isolates belonging to genotype VII, one virus was genotype 1 and other virus are genotype VI. The genetic analysis showed that genotype VII is the dominant group of viruses circulating in Indonesia after 2005, almost Indonesian ND viruses isolated after 1990 also belong to genotype VII. This study identified little differences on the amino acids sequences among viruses in the group of genotype VII. The isolates of ND that we isolated from this study (GTT/11 and SME/13) have dissimilar amino acid sequences of cleavage site on F gene. The GTT/11 isolate have typical velogenic pathotype of NDV. Regardless of the SME/13 isolate based on phylogenetic tree, it belong to genotype VII, but the amino acid sequence of cleavage site have similar motif with I-53. Our result revealed that the I-53 isolate belong to genotype VI, and based on Parade (1987) study, the I-53 isolate was a mesogenic group of viruses. The isolate

of SME/13 was collected from outbreak of ND with high mortality in commercial chicken farm. The recent ND viruses were estimated to be the cause of problem in poultry, so it takes the suitability of the seed vaccine viruses circulating in the field. Our analysis shows that the viruses circulating in Indonesia are viruses that have a high similarity with GTT/11 and SME/13, no new findings on virus which belong to genotypes I and others, because genotype VII is a virus that predominate NDV currently circulating in the field.

There are many kinds of vaccine available in Indonesia. They are used as part of government policy to control ND in Indonesia. The vaccines are imported or produced locally using overseas strains under the government authority. The seed of strains used for local production are still imported strains such as B1, LaSota, F, Komarov (Parade, 1987). Recently, some local vaccine factories used the local strain of NDV to control ND in Indonesia. For years, most of commercial farms in Indonesia have practiced intensive ND vaccinations using both live and inactivated vaccines. In addition, LaSota strain belonging to genotype II is widely used as live vaccine in the field. However, the epidemiological circumstances of ND have been changed by reports of ND outbreaks in the vaccinated chicken flocks. The emergence of new genotypes and antigenic variants of ND infection arose by introduction of the new circulating ND viruses of genotype VII in the farm environment. Despite the fact that significant genotype dissimilarity between seed vaccine and newly identified viruses have been identified, the traditional vaccine of LaSota still could provide certain protection against new genotype VII ND infection because the antigenicity attributes have not changed dra-

matically. However, the high level HI antibody value of flock immunity (up to 8log<sub>2</sub>) maybe required to protect against this new genotype (Liu et al., 2008; Panshin et al., 2002; Yu et al., 2001).

Developing ND seed vaccine in accordance with the field virus circulation should be done to control and reduce the economic impact caused by this disease, so based on this study, it is recommended that the ND virus genotype VII should be used as a new master seed which is expected to induce antibodies and provide good protection against the new field strain of ND virus in Indonesia.

### Conflict of Interests

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

### ACKNOWLEDGEMENTS

We thank to Nana Suryana and Teguh Suyanto for their technical support, assistance and District Livestock Services in Tangerang, Sukabumi and East Java for the field support. This study was supported by DIPA Grant from The Indonesian Agency of Agricultural Research and Development (IAARC), Ministry of Agriculture

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## Full Length Research Paper

# The associated protein screening and identification in *Proteus mirabilis* swarm colony development

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*Proteus mirabilis* colonies exhibit striking geometric regularity, and swarm colony terraces correspond to one swarming-plus-consolidation cycle. Basic microbiological methods and imaging techniques were used to measure periodic macroscopic events in swarm colony morphogenesis. Here, adding a respiratory enzyme indicator, 2,3,5-triphenyltetra-zolium chloride (TTC) into the culture medium, the bacteria on the rings gradually changed into red and the change was not synchronous with the formation of ring. According to this phenomenon, we distinguished five phases as *P. mirabilis* colonize agar surfaces during swarming to investigate the bacteria swarm colony development mechanism by the methods of matrix assisted laser desorption/ionization (MALDI) mass spectrometry and real time fluorescence quantitative-polymerase chain reaction (RTFQ-PCR). Through these observations, we found that *P. mirabilis* swarm colony development was closely related to the gene *fusA*, *fliC1*, *tufB*, *ahpC*, *icd* and *flaB*. The research indicates that the functions of the genes *fusA*, *fliC1* and *tufB* in swarming phase significantly influence the *P. mirabilis* swarming behavior. In consolidation phase, expression of *ahpC* connected to oxidative stress decreased as compared to other phases. The gene *icd* which affected the metabolism had high expression in consolidation phase I and there were more expression from the bacteria on the ring than that from the bacteria between two rings. The increase of *flaB* was concomitant with the development of cell growth from swarming phase to turn red phase II. The morphology of *P. mirabilis* was obviously distinct in different growth phase, and periodically changed. The movement and metabolize way of *P. mirabilis* were diverse in different growth phase. In swarming phase, the swarm colony and antioxidant ability was enhanced, but the functions of respiratory and metabolism was down-regulated. In conclusion, the bacteria fertility ability and respiratory function were enhanced in consolidation phase. These genes related with motion and metabolism played a key role in regulating *P. mirabilis* swarm colony development.

**Key words:** *Proteus mirabilis*, real time fluorescence quantitative-polymerase chain reaction (RTFQ-PCR), matrix assisted laser desorption/ionization (MALDI)-TOF, swarm.

## INTRODUCTION

*Proteus mirabilis* is known for its ability to differentiate between short swimmer cells and swarmer cells, a process crucial for the pathogenesis of these bacteria during urinary tract infections (Alilison and Hughes, 1991; Belas, 1996). It exhibits a striking form of multicellular

behavior, called swarming migration, in which motile vegetative rods growing on solid media differentiate into extremely elongated hyperflagellated swarm cells that undergo rapid and coordinated population migration away from the initial colony (Rahman et al., 2009). In the

spectacular colonies of *P. mirabilis*, a series of concentric rings are developed as the bacteria multiply and swim following a scenario that periodically repeats itself (Czirok et al., 2001). This bacterium can undergo dramatic morphological and physiological changes in response to growth on surfaces or in viscous environments (Allison et al., 1993). These changes are ultimately required to produce the multicellular motile behavior that is characterized by flagellum-assisted swarming motility over nutrient agar media.

*P. mirabilis* swarm colony terrace corresponds to one swarming-plus-consolidation cycle, which distinguished three initial phases (lag phase, first swarming phase and first consolidation phase) followed by repeating cycles of subsequent swarming plus consolidation phases (Darnton et al., 2010). The lag phase is the differentiation of the first swarmer cell perimeter in preparation for swarming, ends when the first swarmer rafts emerge from the colony perimeter. The first swarming phase is characterized by relatively independent movement of swarmer cell rafts which tend to coalesce into radially oriented tubes, ends when migration which ceases and the leading edge remains stationary. As the first swarming phase comes to an end, a wave of cell multiplication and thickening of the newly colonized areas begins adjacent to the perimeter of the inoculation spot. The second and following swarming phases are more coherent than the first swarming phase, ends when the swarming front ceases to advance rather synchronously along its entire perimeter. The second and following consolidation phases form a uniform thickening of the newly formed terrace instead of spreading wave of multiplication (Rauprich et al., 1996).

Cell density, surface contact, inhibition of flagellar rotation and cell-to-cell signalling all provide critical stimuli, and close cell alignment and the production of secreted migration factors facilitate mass translocation (Armbruster and Mobley, 2012). The first pivotal stimulus of swarm cell differentiation is surface contact. Differentiation is induced when viscosity of the growth medium is increased, or when flagella are tethered with antibodies. Alavi and Belas (2001) reported that hyperflagellation is also the most prominent feature of swarm cells, and differentiation requires efficient flagella assembly. Cell density is critical to swarming as the duration of the lag phase that precedes *P. mirabilis* migration is strongly influenced by inoculum density. Swarming cells also require extracellular components (e.g. polysaccharide and surfactants) that allow mass migration of differentiated cells over difficult terrain. AL-2, putrescine and cyclic dipeptide have also been proposed as extracellular signals that are capable of mediating cell-cell communication (Schneider et al., 2002; Sturgill et al.,

2002; Sturgill and Rather, 2004).

In the past 20 years, there has been a significant acceleration in our understanding of *P. mirabilis* as it pertains to the process of migration and differentiation, which refer to regulation of the gene, lipopolysaccharide and peptidoglycan synthesis, bacterial division, ATP production, putrescine biosynthesis, proteolysis and morphological changes (Allison and Hughes, 1991a,b; Williams and Schwarzhoff, 1998). The process of elongation takes place with only a slight increase in cell width and is due to an inhibition in the normal septation mechanism, although the molecular mechanism of inhibition is not known. Elongation of the swarmer cell can give rise to cells 60 to 80  $\mu\text{m}$  in length. During this process, DNA replication proceeds without significant change in rate from that in the swimmer cell (Gmeiner et al., 1985). Not surprisingly, the rate of synthesis of certain proteins, e.g. flagellin, the protein subunit of the flagellar filament, is altered markedly in the swarmer cell (Armitage et al. 1979; Armitage, 1981; Falkinham and Hoffman, 1984). The differentiation process leading to the development of a swarmer cell involves between 25 and 50 genes that are coordinately expressed (Belas, 1994; Mobley and Belas, 1995). Thus, since *P. mirabilis* is frequently associated with urinary tract infections, particularly in patients with chronic urinary catheterization, knowledge of the regulation of swarmer cell gene expression is crucial to understanding the pathogenesis of this organism.

While several signals are believed to induce differentiation (for example, surface contact, cell density and amino acids) the pathways of signal integration are still poorly understood, in particular the apparent surface contact-sensing by flagellar filaments and the basis of the cell-cell communication assumed to underlie coordinated migration. Our previous studies on some preliminary work shows the influence of strain, pH, the concentration of agar, and the inoculate way on growth. In our study, we observed the changes of modulative gene in the process of swarm colony development. Although many attempts have been made to explain the mechanism of the swarming in *P. mirabilis*, including slime production and chemotaxis, the process of swarmer cell differentiation and multicellular swarming motility remains elusive. In our study, the experiment aimed to investigate the proteins in five phases of *P. mirabilis* by the methods of RTFQ-PCR, which provides a new experimental evidence for clarifying the mechanisms of the swarm colony development.

## MATERIALS AND METHODS

### Strain, media, and culture conditions

Clinical isolates of *P. mirabilis* HI4320 were obtained from the

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microbiology laboratory of the Third Military Medical University (Chongqing, China). We routinely grew *P. mirabilis* colonies on LB agar containing 0.1% glucose, 1% tryptone and 0.5% yeast powder. It was grown in LB medium at 37°C. To obtain homogeneous populations of differentiating bacteria, 0.2% 2,3,5-triphenyl four azole nitrogen chloride (TTC) was spread onto a LB (2%) agar plate, and the latter was incubated for various periods (from 0 to 24 h).

### Bacterial sample preparation

An overnight culture of cell was spot inoculated in the center of each LB agar plates. Following incubation at 37°C for 12 h, cells were scraped off the agar surface with bacterial rings. We divided the growth cells into 5 phases: swarming phase, consolidation phase I (cells on the ring), consolidation phase II (cells between two rings), turn red phase I (cells on the ring) and turn red phase II (cells between two rings). The cells were collected by the inoculating loop and suspended in 1 mL phosphate-buffered saline (PBS).

### Extraction of total protein

Total protein was extracted from bacteria by using the Bacterial Protein Extraction Kit (Sangong, Shanghai, China) which was designed for the extraction of biologically active soluble proteins and high purity inclusion bodies from bacterial cells. Briefly, cells were collected and immediately washed completely with iced PBS (pH=7.2) at 4°C (centrifuging at 5000  $\times$ g for 10 min). After removing the media and washing the pellet with PBS, the cells were re-suspend pellet in 1 ml of 1 $\times$ cell lysis buffer. Adding 40  $\mu$ l phenylmethylsulfonyl fluoride (PMSF) and 80  $\mu$ l lysozyme, the cell suspension were incubated at 37°C for 30 min and then on a rocking platform for 10 min. The mixture was added 20  $\mu$ l DNaseI/RNase and incubation was continued on a rocking for another 10 min at 37°C. Finally, the insoluble debris were removed by centrifugation at 3000  $\times$ g for 30 min at 4°C and then collecting the supernatant in a fresh tube. The bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) was used according to the manufacturer's instructions using the microplate procedure (20  $\mu$ l sample/200  $\mu$ l BCA working reagent; 37°C/30 min; 562 nm).

### SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by use of slab gels consisting of a 12% acrylamide gradient resolving gel and 5% concentrating gel. The samples were placed at -20°C. Prior to electrophoresis, samples were vortexed, and 1 $\times$ loading buffer were added to adjust the supernatant to an equal volume of 50  $\mu$ g. The samples were boiled at 100°C for 5 min, and centrifuged for 5 s. The samples were loaded on a 5% concentrating gel and run until the bromophenol blue was at the very bottom of the gel at 50 V for 30 min and 100 V for 90 min. The gels were stained with Coomassie brilliant blue and destain. The molecular weights of proteins were estimated from their mobilities relative to a set of commercially available proteins.

### MALDI-TOF MS analysis

According to the results of SDS-PAGE, we cut off the corresponding protein bands and sent the samples to the Burn Department of Southwest Hospital. The matrix assisted laser desorption/ionization (MALDI) mass spectrometry was employed. The differentially expressed proteins were digested with trypsin. We

selected eight bands and subjected them to matrix-assisted laser desorption/ionization time of flight mass spectrometry followed by searching ncbi (NCBI) database to identify these proteins (Wu et al., 2009). All MALDI-TOF mass spectrometry results were compared with results from conventional methods, and discrepancies were resolved by 16S rRNA gene sequencing.

### RTFQ-PCR analysis

According to the MALDI-TOF results, the corresponding protein mRNA levels were verified with real-time fluorescence quantitative PCR (RTFQ-PCR) analysis for *P. mirabilis* by using a Real-time Quantitative PCR Detecting System (biosystems, USA). PCR was performed in a 20  $\mu$ l reaction volume that contained ultrapure water, PCR buffer, 50 mM MgCl<sub>2</sub>, 2.5 mM dNTPs, 0.5  $\mu$ l Upstream primer, 0.5  $\mu$ l Downstream primer, 0.3  $\mu$ l SYBR, 0.2  $\mu$ l Taq DNA Polymerase and 1  $\mu$ l cDNA. The PCR protocol was as follows: initial denaturation for 2 min at 95°C, 40 cycles at 95°C for 10 s, 60°C for 30 s, and 60°C for 45 s. Real-time PCR was performed on the corresponding cDNA synthesized from each sample. We choose 16SrRNA as the internal control gene to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. The data were analyzed by  $\Delta\Delta C_T$  method, where,  $\Delta\Delta C_T = (C_T, Target - C_T, Actin)_{Time\ x} - (C_T, Target - C_T, Actin)_{Time\ 0}$  (Kenneth and Thomas, 2001).

## RESULTS

### Culture results

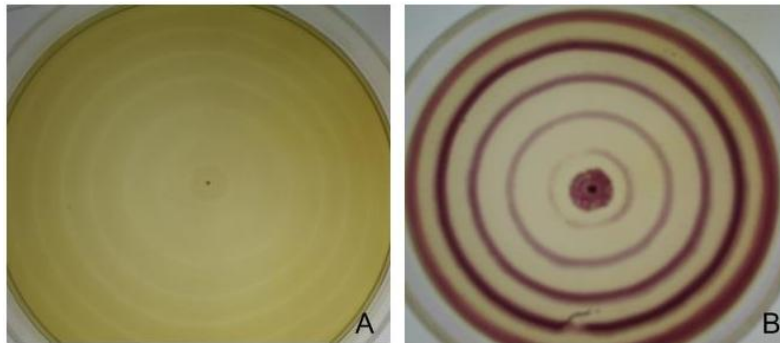
*P. mirabilis* in LB medium was incubated at 37°C for 12 h, cells were scraped off the agar surface with bacterial rings, while adding 1% TTC aqueous into LB medium, the color of bacteria on the concentric rings was changed to red (Figure 1). However, the color of bacteria between two rings was not change.

### SDS-PAGE analysis

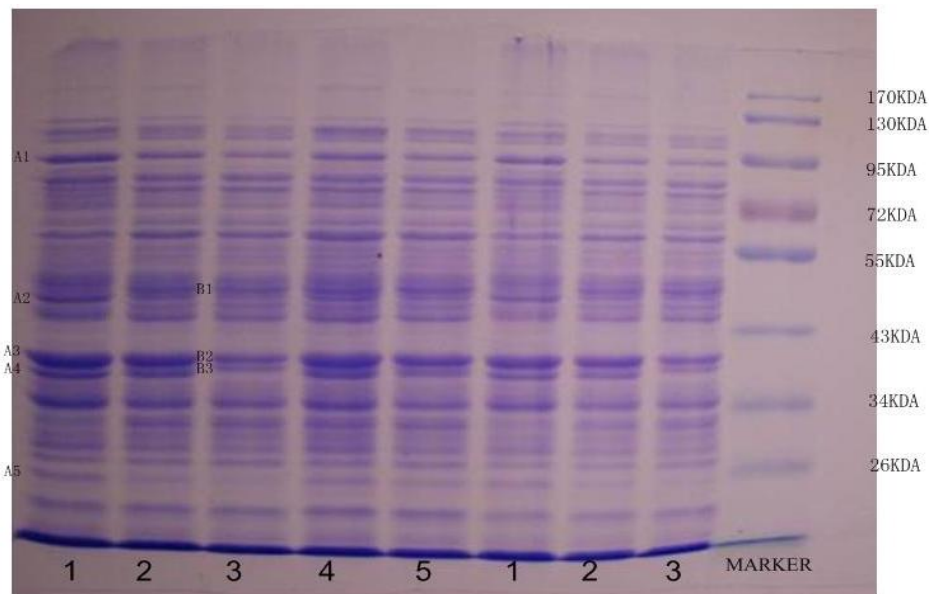
Denatured polyacrylamide gel electrophoresis was employed to assess the differences of protein pattern in the experimental samples that belong to *P. mirabilis*. In SDS-PAGE analysis, a total of eight protein bands were determined (Figure 2). Five bands with molecular weights of 95 - 130 kDa (A1), 43 - 55 kDa (A2), 34 - 43 kDa (A3 and A4) and 26 kDa (A5) in swarming phase were all putative different proteins as compared to the other phases. Results show that the contents of protein (A1, A3 and A4) were decreased regularly in groups I, II, and III while the contents of protein in Group IV and Group V were increased. In addition, in Figure 2, the Group II~V samples corresponded to B1 band while the Group I sample did not have. The result of SDS-PAGE analysis revealed that the absence and/or presence of bands were observed in the five phases.

### MALDI-TOF analysis

These eight proteins bands treated by tryptic in-gel



**Figure 1.** The periodic growth patterns of *P. mirabilis*. A: Without TTC in the LB culture medium. B: With TTC in the LB culture medium.



**Figure 2.** The SDS-PAGE of bacteria complete protein. Lane 1, Swarming phase; Lane 2, bacteria on the ring in consolidation phase; Lane 3, Bacteria between the rings in consolidation phase; Lane 4, bacteria turn red and in the ring; Lane 5, bacteria between the rings turned red.

digestion were characterized by MALDI-TOF MS and peptide mass finger printings of all were obtained. A novel protein or polypeptide was identified by database retrieval. NCBI was used to identify nine proteins of *P. mirabilis* HI4320 based on the MS Search Score greater than 70. The result is shown in Table 1.

### RTFQ-PCR analysis

As shown in Table 2, we chose six target proteins including alkyl hydroperoxide reductase (*ahpC*), fructose biphosphate aldolase (*fbaB*), isocitrate dehydrogenase (*icd*), EF-G (*fusA*), EF-Tu (*tufB*), and flagellin 1 (*fliC1*) to

do the RTFQ-PCR analysis. Primers were designed for these six genes (Table 2 for protein name, gene name, primer sequences). It was generally accepted that gene-expression levels should be normalized by a carefully selected stable internal control gene. The expression level of 16sRNA is shown in Table 3. Three genes expression levels (*fusA*, *fliC1* and *tufB*) which involved bacterial growth were obtained. The expression levels of *fusA* (Figure 3) and *fliC1* (Figure 4) in swarming phases were higher than other tested phases, and the level of *fusA* gradually reduced from Group I to Group V, while the level of *fliC1* reduced no regularity. An opposite trend was observed in the expression of *tufB* (Figure 5), the *tufB* levels in swarming phase were lower than the

**Table 1.** Summary of differentially expressed proteins of *P. mirabilis* by MALDI-TOF analysis.

Run name	Protein name	Mean peptide spectral intensity	AA coverage (%)	MS search score	Distinct peptides
A1	Elongation factor G	9.85e+007	48	430.50	24
A1	Formate acetyltransferase	3.62e+007	10	89.65	5
A2	Elongation factor TU	2.61e+008	50	246.80	15
A2	Phosphopentomutase	1.19e+008	21	114.67	6
A3	Flagellin 1	1.99e+008	61	322.32	19
A4	Flagellin 1	2.89e+007	26	106.45	6
A4	Outer membrane porin	3.02e+007	20	85.03	6
A5	Alkyl hydroperoxide reductase	5.89e+006	34	81.46	5
B1	Isocitrate dehydrogenase	6.18e+007	41	203.95	13
B1	Phosphopentomutase	2.12e+007	14	81.32	4
B2	Flagellin 1	8.21e+007	57	282.80	17
B3	Fructose-bisphosphate aldolase	1.63e+007	26	94.84	5
B3	Flagellin 1	2.44e+007	14	72.16	4

**Table 2.** The primer information of RTFQ-PCR analysis for *P. mirabilis* mRNA levels.

Target protein	Target gene name	Primer
Alkyl hydroperoxide reductase	<i>ahpC</i>	CTTCTGGCCAATGGACTTCA ACGCCATGCGTTATGTACGA
Fructose bisphosphate aldolase	<i>fbaB</i>	GCCGGATGTAAGTGTGTTGC GTCGCACCTACAGCAACAGC
Isocitrate dehydrogenase	<i>icd</i>	AGGTGGTGGTATCCGTTTCAT CCTACTCAATACCGGCATAGA
EF-G	<i>fusA</i>	TCACATCCGCTGCAACTACT GAGGCTGAACACCACCAACT
EF-Tu	<i>tufB</i>	CCATTGAAGCCGGATGTAAC GTCGCACCTACAGCAACAGC
Flagellin 1	<i>fliC1</i>	AGTGCAATCGAGCGTCTGTC TCGTTTCGCATTACGTGAAGC

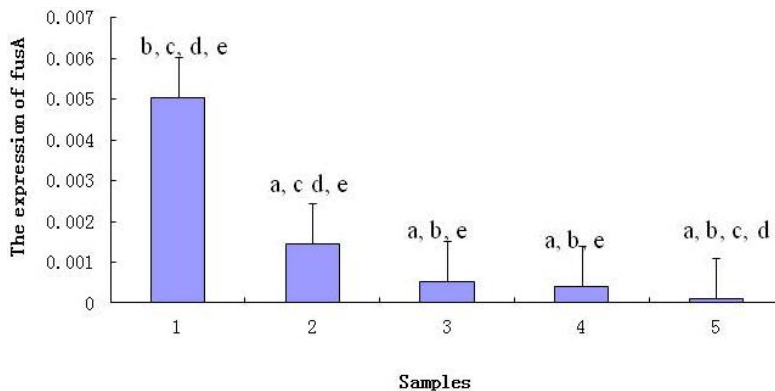
**Table 3.** The expression of 16S rRNA gene.

Sample name	Target name	Ct	Ct Mean
1	16srRNA	13.64741	13.74631
	16srRNA	13.78528	
	16srRNA	13.80623	
2	16srRNA	13.51925	13.48805
	16srRNA	13.46205	
	16srRNA	13.48284	
3	16srRNA	13.66578	13.61869
	16srRNA	13.57320	
	16srRNA	13.61710	
4	16srRNA	13.35785	13.44047
	16srRNA	13.45795	
	16srRNA	13.50561	
5	16srRNA	13.65908	13.6018
	16srRNA	13.86087	
	16srRNA	13.28546	

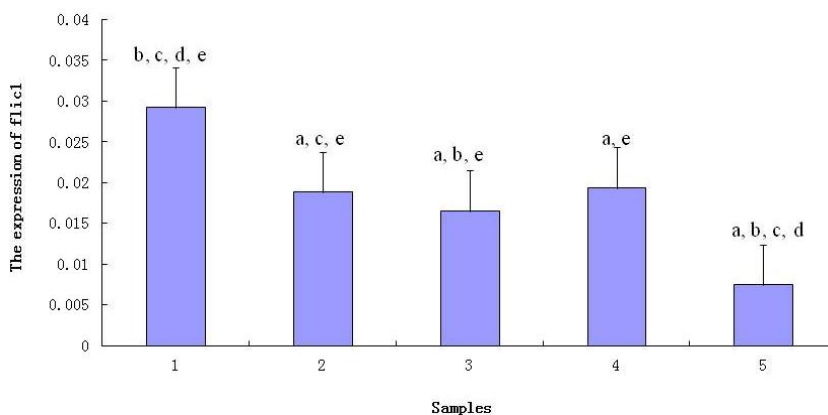
other phases. The expressed product of *ahpC* (Figure 6) gene is alkyl peroxide reductase which acts on the oxidative stress. In the swarming phase, the level of *ahpC* had the highest expression amount, the other four phases showed tendency to increase. These genes (*icd* and *fbaB*) were associated with bacterial metabolism, in which isocitrate dehydrogenase encoded by *icd* gene was the rate-limiting enzyme of tricarboxylic acid cycle (TAC), and diphosphofructose enzyme encoded by *fbaB* gene was the rate-limiting enzyme of glycolysis. In this paper, the expression levels of *icd* (Figure 7) and *fbaB* (Figure 8) in swarming phase were both significantly lower than the other phases.

## DISCUSSION

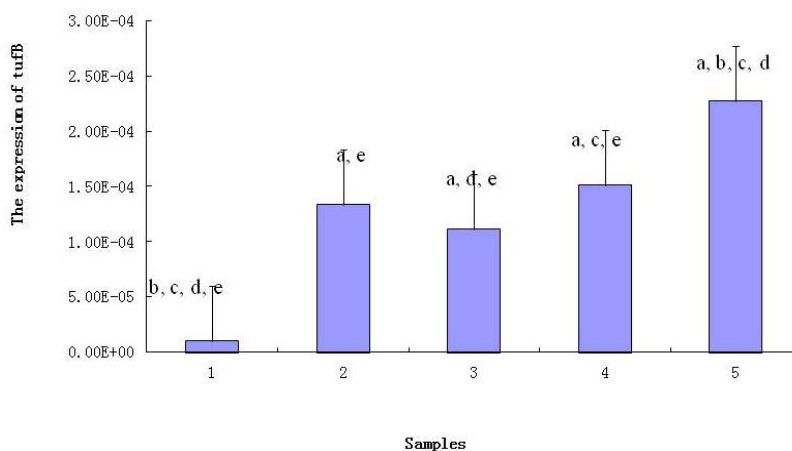
The mechanism of the swarm colony development on protein expression of *P. mirabilis* was investigated using a



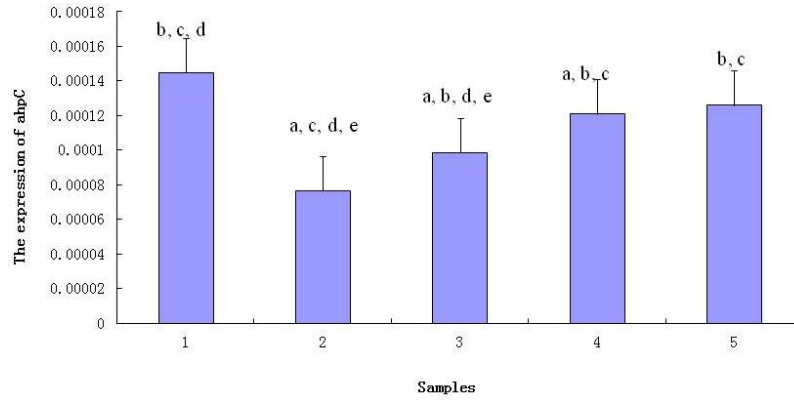
**Figure 3.** The expression of *fusA*. a: Compared with group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with Group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



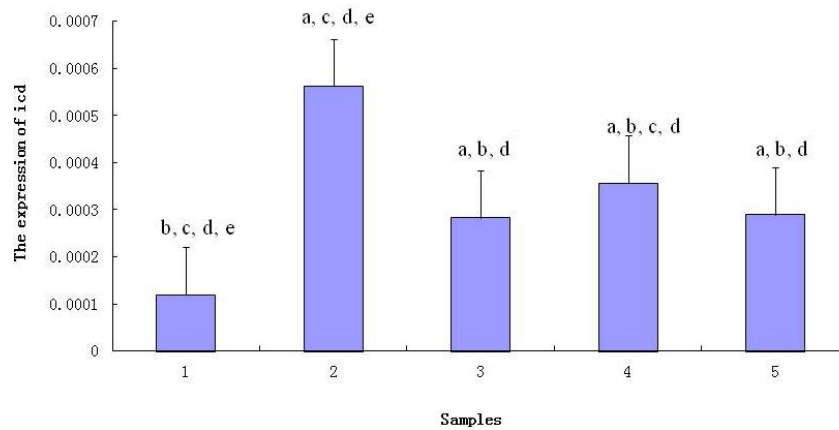
**Figure 4.** The expression of *fliC1*. a: Compared with group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



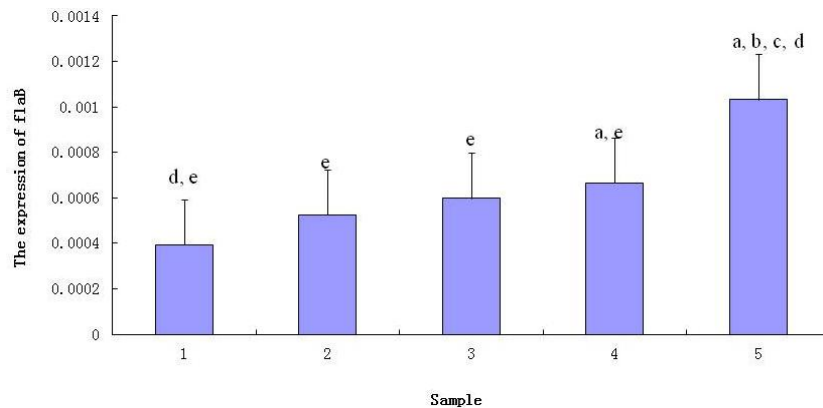
**Figure 5.** The expression of *tufB*. a: Compared with Group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



**Figure 6.** The expression of *ahpC*. a: Compared with group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



**Figure 7.** The expression of *icd*. a: Compared with group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



**Figure 8.** The expression of *flaB*. a: Compared with group 2 ( $p < 0.05$  or  $p < 0.01$ ); b: compared with group 3 ( $p < 0.05$  or  $p < 0.01$ ); c: compared with group 4 ( $p < 0.05$  or  $p < 0.01$ ); d: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ); e: compared with group 5 ( $p < 0.05$  or  $p < 0.01$ ).



proteomic method. The genetic studies were successful in identifying key events leading to the swarming behavior differentiation, such as the regulation of master operon *flhDC* and its products, which was central to the control of cell division, as well as the transcriptional regulation of flagellar biogenesis. However, genetics did not reveal the dynamics of interactions involved in the long-range correlations which occur during swarming (Claret and Hughes, 2000; Chilcott and Hughes, 2000). The eightfold increase in *fliC1* expression during induction of swarmer cell differentiation is less than expected from observations of the increase in flagellar filaments when swimmer cells differentiate into swarmer cells (Robert, 1994). In our study, the expression of *fliC1* gene in swarming phase was increased when compared with the other phases. On the basis of the evidence presented, only *fliC1* was actively expressed in cells, the transcription of *fliC1* results in the synthesis of flagella composed solely of *fliC1*. It is possible related to the posttranslational control which was involved in controlling the amount of *flic1* synthesized during swarmer cell differentiation. With the increase of *fliC1* gene, the number of flagellin 1 encoding by *fliC1* was increased, which was an important substance of flagella. Far away from the swarming phase, the expression of *fliC1* was the lowest, the content of flagellin and synthesis of flagellar were relevantly reduced. The results thoroughly agree with our previous bacterial flagella staining (unpublished).

*P. mirabilis* is a facultative aerobe, with normal growth and under conditions of oxygen and no oxygen, the growth mode did not change. In recent years, scholars both at home and abroad have done a great deal of researches on the metabolism and energy sources, which showed that there were numerous differences in physiology of swarmer cells as compared to non-swarmer cells. For example, swarmer cells have reduced amino acid uptake and have increased sensitivity to hydrophobic antibacterial agents, suggesting that reorganization of the outer membrane might affect active transport processes and protective properties (Armitage et al., 1975; Falkinham and Hoffman, 1984). During swarming, the incorporation of precursors into DNA, RNA, proteins and the rate of oxygen uptake were also reduced (Armitage et al., 1979). Then, at the end of the swarm period, macromolecule synthesis and oxygen uptake were restored to levels equivalent to pre-swarming levels. Furthermore, a study showed that membrane vesicles from swarm cells have reduced rates of NADH, malate and succinate respiration (Falkinham and Hoffman, 1984). Interestingly, some researchers pointed out that the energy required during swarming was generated by fermentation (Himpsl et al., 2008). However, fermentation would be much less energetically favorable for fueling the flagellar motor as compared to membrane respiration. Christopher et al., (2012) found that swarming bacteria use a complete aerobic tricarbo-

xylic acid (TCA) cycle but not employ oxygen as the terminal electron acceptor. They previously identified two *P. mirabilis* transposon mutants disrupting genes encoding proteins (pyruvate dehydrogenase and succinate dehydrogenase) in the oxidative TCA cycle that each displayed an aberrant swarming phenotype, which suggested a close link between the TCA cycle and swarming motility.

Proteomics analysis of proteins with different expressions reveals that most of the identified proteins were metabolism related. In this study, we screened isocitrate dehydrogenase and fructose biphosphate aldolase which are metabolism-related proteins, as research object (Thorsness and Koshland, 1987). Isocitrate dehydrogenase was an important rate-limiting enzyme of the TCA which is encoded by *icd* and could convert isocitrate to alpha ketoglutarate. It was revealed by RT-PCR that *icd* gene expression had minimum quantity in the swarming phase. Results indicate that the current primary coverage of bacterial activity in this phase was forward movement, rather than the metabolism. In agreement with the TTC in the LB medium, bacterial color was pale yellow in swarming phase, indicating the activity of succinate dehydrogenase was also low. Succinate dehydrogenase and isocitrate dehydrogenase are the rate-limiting enzyme of TCA, a small amount of metabolism at this time was only for flagella to rotate to provide energy. In addition, no matter which bacteria is on the ring in consolidation phase (group 2) and bacteria turn red in the ring (group 4), expression of *icd* gene were higher than bacteria out of the ring (groups 1, 3 and 5). Bacteria on the rings were considered as in consolidation phases, which showed that *P. mirabilis* aerobic respiration in this stage was restored to normal, and the ability of metabolism was enhancement. Simultaneously, red rings appeared in the medium supplemented with TTC and this led to an increase in succinate dehydrogenase concentration.

Corresponding to the periodic motion, bacterial metabolism was a cyclical change. Fructose-bisphosphate aldolase was a rate-limiting enzyme of glycolysis which is encoded by *fbaB* and could catalyze the formation of glyceraldehyde 3-phosphate and D-glyceraldehyde 3-phosphate from D-fructose 1, 6-bisphosphate. In our work, level of *fbaB* gene expression was minimum in swarming phase, and more away from swarmer cells, more *fbaB* gene were expressed, the result also confirmed this view that the bacteria metabolism ability was reduced and movement ability was enhanced in swarming phase.

Aerobic metabolism leads to oxidative stress from generation of reactive oxygen intermediates (ROI). *AhpC* gene which encodes alkyl peroxide reductase protected both bacterial and human cells against reactive nitrogen intermediates (Chen et al., 1998). In the swarming phase, the level of *ahpC* had the highest expression amount, the other four phases from groups 2 to 5 showed a tendency



to increase. The results indicated that in swarming phase oxygen was a negative factor on bacteria. As mentioned before, the metabolism of this bacterial phase do not need oxygen, in such a bad environment; bacteria were moving but not for reproduction process. These properties of *P. mirabilis* and tumor cells were just different in approaches but equally with satisfactory result, and metabolism of anaerobic respiration was the most primitive in tumor cells. When the external environment was not conducive for the bacteria growth, bacteria would be reversion of anaerobic respiration. However, the intrinsic relation should be further explored.

Besides metabolism-related proteins, genetic information processing related proteins are discovered during acid adaptation. Elongation factor Tu (EF-Tu) and elongation factor G (EF-G) are translation-related proteins and are needed for protein synthesis, they are structural homologues and share near-identical binding sites on the ribosome, which encompass the GTPase-associated center (GAC) and the sarcin-ricin loop (SRL) (Yu et al., 2007; Zavialov and Ehrenberg, 2003). The SRL is fixed structure in the ribosome and contacts elongation factors in the vicinity of their GTP-binding site. In contrast, the GAC is mobile and we hypothesized that it interacts with the alpha helix D of the EF-Tu G-domain in the same way as with the alpha helix A of the G'-domain of EF-G. The mutual locations of these helices and GTP-binding sites in the structures of EF-Tu and EF-G are different. Thus, the orientation of the GAC relative to the SRL determines whether EF-G or EF-Tu will bind to the ribosome (Sergiev et al., 2005). EF-Tu and EF-G are GTP-dependent elongation factors, and they are responsible for binding of aminoacyl tRNA to rRNA and translocation, respectively. Another interesting discovery is EF-Tu (Sanderson and Uhlenbeck, 2005; Furness et al., 1997). This protein is encoded by *tufB* and can promote the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis (Swiss-Prot) (Karring et al., 2003). In this study, the RT-PCR analysis showed that the expression of *fusA* in swarm phase was significantly increased and more than 3 to 14 times of other phases. Furthermore, the more close to the swarming phase in the process of bacterial growth, the higher the amount of *fusA* expressed. However, the expression of *tufB* was just the opposite. This indicated that the swarming behavior was closely related to the *fusA* and *tufB* genes, even there exists competition relationship in the process of *P. mirabilis* swarming development (swarm cells to swimmer cell). Especially differentiation processes might necessitate large amount of protein synthesis; it referred to cell growth and inevitably lead to changes of gene expression in protein synthesis. This results showed large increase in *fusA* expression, which indicated that it mainly participated in the synthesis of cell growth and flagella multiplication proteins. While the expression of *tufB* was significantly raised in consolidation phase, it showed that

*tufB* participated in the synthesis of bacterial reproduction proteins. We demonstrated that *fusA* and *tufB* can positively regulate *P. mirabilis* swarming behavior whose mechanisms need to be further clarified.

In conclusion, *P. mirabilis* swarm colony development was closely related to the proteins of EF-Tu, EF-G, flagellin 1, fructose bisphosphate aldolase, isocitrate dehydrogenase and alkyl hydroperoxide reductase in these five phases. The movement and metabolism mode of *P. mirabilis* was diverse in different growth phase. These genes related with motion and metabolism played a key role in regulating *P. mirabilis* swarm colony development.

### Conflict of interests

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

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## Full Length Research Paper

# Involvement of clathrin and $\beta$ -arrestins in *Aggregatibacter actinomycetemcomitans* endocytosis of human vascular endothelial cells

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Phosphorylcholine (PC) has been reported to help PC-bearing *Aggregatibacter actinomycetemcomitans* to gain access to the general circulation via interaction with the platelet-activating factor receptor (PAFR) on endothelial cells. However, the underlying mechanism remains unclear. Here, we found that inhibition of clathrin and  $\beta$ -arrestins blocked bacterial PC binding to PAFR which significantly attenuated *A. actinomycetemcomitans* invasion of human vascular endothelial cells (HUVEC). These results demonstrated the involvement of clathrin and  $\beta$ -arrestins in PC-bearing *A. actinomycetemcomitans* invasion of HUVEC.

**Key words:** *Aggregatibacter actinomycetemcomitans*, clathrin,  $\beta$ -arrestins, human vascular endothelial cells.

## INTRODUCTION

*Aggregatibacter actinomycetemcomitans* is a significant periodontal pathogen implicated in aggressive periodontitis and adult periodontitis (Asikainen et al., 1997; Meyer and Fives-Taylor, 1997; Zambon, 1985). This Gram-negative bacterium has been shown to adhere and penetrate epithelial and endothelial cells (Meyer et al., 1996; Schenkein et al., 2000).

Phosphorylcholine (PC) has been reported as a viru-

lence factor in a number of pathogenic prokaryotes, including Gram-positive bacteria *Streptococcus pneumoniae* and the Gram-negative species *Haemophilus influenzae* (Harnett and Harnett, 1999). PC not only plays an important role in *S. pneumoniae* adherence and invasion of host cells (Cundell et al., 1995), but also enhances *H. influenzae* biofilm maturation (Hong et al., 2007). Recent studies have identified *A. actinomycetemcomitans* as

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**Abbreviations:** PC, Phosphorylcholine; PAFR, platelet-activating factor receptor; HUVEC, human vascular endothelial cells; GPCRs, G proteins coupled receptors; MDC, monodansylcadaverine; CPZ, chlorpromazine; MOI, multiplicity of infection.

**Table 1.** Plasmids of  $\beta$ -arrestin-1/-2 siRNA.

Plasmid	Sequence
ARRB1-3	5'- GGATCCTGAGTATCTCAAAGATTCAAGAGATCTTTGAGATACTCAGGATCCTT -3'
ARRB2-1	5'- GGACCAGGGTCTTCAAGAAGTTTCAAGAGAAGTTCTTGAAGACCCTGGTCCTT -3'
ARRB-NC	5'- GTTCTCCGAACGTGTACCGT <u>CAAGAGATT</u> ACGTGACACGTTCCGGAGAA TT -3'

**Table 2.** Primers used in real time PCR.

Primer	Sequence	Amplified region (bp)
ARRB1-F	5'-ACACGGGTGTATTACATTCCGG-3'	88
ARRB1-R	5'-CCAGTGAGGTGGGAAGAGC-3'	
ARRB2-F	5'-CCCTCAATGTAAATGTCCACG-3'	86
ARRB2-R	5'-GATGTCGGCGTACTGTCTCAC-3'	
$\beta$ -actin-F	5'-CCCTGGCACCCAGCAC-3'	70
$\beta$ -actin-R	5'-GCCGATCCACACGGAGTAC-3'	

variable in reaction with PC specific monoclonal antibodies, TEPC-15 (Schenkein et al., 2000; Purkall et al., 2002). PC helps PC-bearing *A. actinomycetemcomitans* to invade human vascular endothelial cells via the platelet-activating factor receptor (PAFR) as well as *S. pneumoniae* and *H. influenza* (Schenkein et al., 2000; Cundell et al., 1995; Swords et al., 2000).

As typical of G proteins coupled receptors (GPCRs), PAFR activation by PC could be regulated by  $\beta$ -arrestins (McLaughlin et al., 2006; Eckels et al., 2009).  $\beta$ -Arrestins could bind to activated GPCRs and clathrin to promote the clathrin-mediated endocytosis and transcytosis (Shenoy and Lefkowitz, 2011; Gyombolai et al., 2013). The role of  $\beta$ -arrestins in inflammation process has been well known by regulation of chemokine responsiveness (Vroon et al., 2006; Cattaruzza et al., 2013; Porter et al., 2010). It has been reported that clathrin and  $\beta$ -arrestin-1 could mediate *S. pneumoniae* and *Acinetobacter baumannii* invasion of epithelial cells (Radin et al., 2005; Smani et al., 2012). However, there are no studies about clathrin and  $\beta$ -arrestins in *A. actinomycetemcomitans* internalization by epithelial or endothelial cells. In this work, we aimed to study whether clathrins and  $\beta$ -arrestins participated in endocytosis of *A. actinomycetemcomitans*.

## MATERIALS AND METHODS

### Bacterial strains and media

*A. actinomycetemcomitans* strains HK1651 and ATCC29523 were purchased from ATCC Bioresource Center (USA). HK1651 was identified as PC-positive strain and ATCC29523 was PC-negative strain before. The brain heart infusion medium was used as the liquid medium and Tryptic soy agar (TSA) was used as the solid medium (Huankai Microbial Sci. &Tech. Co. Let, Guangdong, China). The *A. actinomycetemcomitans* strains were grown in humidified 5% CO<sub>2</sub> atmosphere at 37°C.

### Cell culture

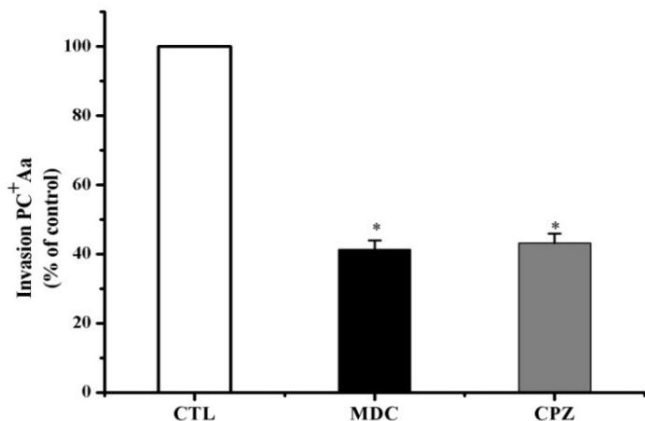
The human umbilical vein endothelial cells (HUVEC) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM medium, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). Cells were incubated at 37°C, 5% CO<sub>2</sub>, 95% air humidified atmosphere.

### Plasmids and transfection

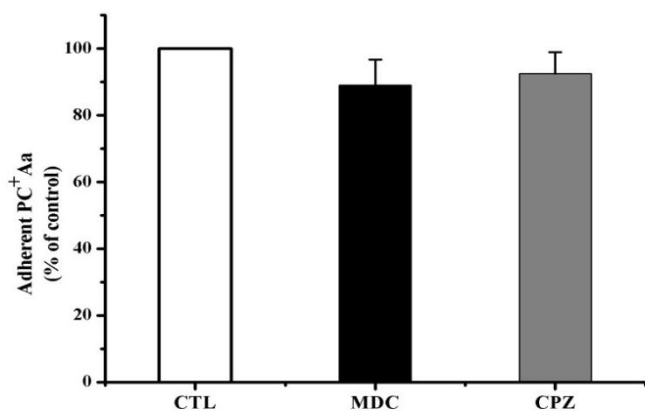
The double-stranded siRNAs for  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 were chemically synthesized and sequenced by Shanghai GenePharma Co., Ltd. The sequences of  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 siRNAs used in this study are listed in Table 1. HUVEC was transfected with the plasmids using Lipofectamine™ LTX and PLUS™ Reagent (Invitrogen, USA) according to the manufacturer's instruction. Briefly, 0.5  $\mu$ g of DNA in 100  $\mu$ l of Opti-MEM® I Reduced Serum Medium (Hyclone, USA) without serum were mixed with 1.25  $\mu$ l of Lipofectamine™ LTX and 0.5  $\mu$ l PLUS™ Reagent. The mixture was then added to the wells containing HUVEC at 37°C for 24 h. After the transfection, the cells were observed under an inverted fluorescence microscope and the real-time reverse-transcription PCR was used to examine the  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 expression.

### Real-time reverse-transcription PCR

The total cellular RNA was extracted with TRIzol® (TaKaRa Biotechnology Co., Ltd.) and reverse transcribed into cDNA using the ExScript RT Reagent Kit (TaKaRa Biotechnology Co., Ltd.). Real-time PCR was performed by employing the SYBR® Premix Ex Taq™ Kit (TaKaRa Biotechnology Co., Ltd.) and was run on the ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The  $\beta$ -actin was used as an internal control. Primers used in this study are listed in Table 2. The thermal cycling was initiated with a first denaturation step of 30 s at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 90 s. Melting curve analysis was performed and concentration values were measured. The detected cycle threshold (Ct) values were interpolated into the standard curves of the plasmid constructs, and the mRNA expression in the samples was calculated by  $2^{-\Delta\Delta Ct}$ .



**Figure 1.** Clathrin inhibitors block PC positive *A. actinomycetemcomitans* invasion of HUVEC. Confluent HUVEC monolayers on the 24-well plate were treated with the indicated concentrations of MDC and CPZ for 30 min before addition of the bacteria. Results are presented as relative invasion of PC positive *A. actinomycetemcomitans* (%). Data are means  $\pm$  standard deviation of five independent experiments done in triplicate (\* $P < 0.05$ ).



**Figure 2.** Relative adhesion of PC positive *A. actinomycetemcomitans* (%) after clathrin inhibitors were blocked. The adhesion examination was carried out as described earlier. Data are means  $\pm$  standard deviation ( $P < 0.05$ ). The adhesion showed no significant difference between pretreated and unpretreated groups.

#### Bacterial invasion and adhesion assays

To determine the role of clathrin in *A. actinomycetemcomitans* internalization by HUVEC, the cells were pretreated with monodansylcadaverine (MDC) or chlorpromazine (CPZ) for 30 min prior to infection with PC positive and negative *A. actinomycetemcomitans* strains (OD<sub>650</sub>=0.9) at a multiplicity of infection (MOI) of 100. After 4 h incubation at 37°C, the invasion assay was performed as described by Schenkein et al. (2000).

Briefly, the monolayers were incubated with gentamicin (50 mg/ml) for 2 h to kill bacteria external to the HUVEC. Then the monolayers were lysed and bacteria were plated to enumerate total cell-associated bacteria. For adhesion assay, the number of total cell-associated bacteria was determined as above without the gen-

tamicin step.

To study the role of  $\beta$ -arrestins in *A. actinomycetemcomitans* internalization by HUVEC, PC positive and negative *A. actinomycetemcomitans* strains were added to the confluent monolayers of HUVEC transfected or not with siRNA of  $\beta$ -arrestin-1,  $\beta$ -arrestin-2 and control. The adhesion and internalization assays were done as described above.

#### Statistical analysis

The group data are presented as mean  $\pm$  S.D. A student *t*-test was used to determine the differences between means. The difference was considered significant at  $P < 0.05$ . The SPSS (version 13.0) statistical package was used (SPSS Inc., Chicago, IL).

## RESULTS

### Clathrin role in *A. actinomycetemcomitans* internalization by HUVEC

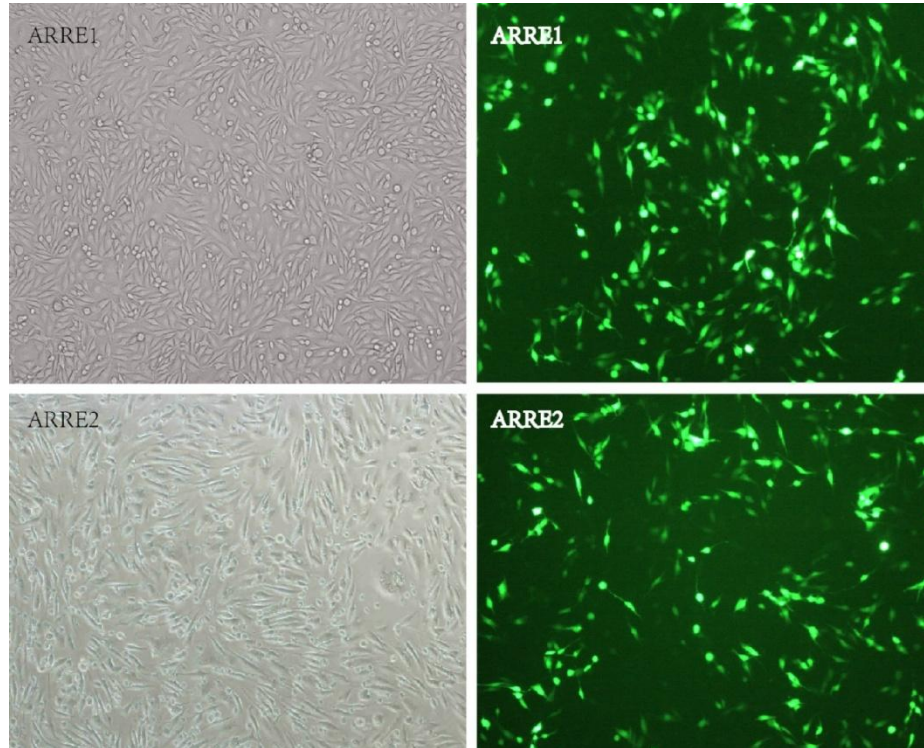
To investigate whether the clathrin was involved in the *A. actinomycetemcomitans* endocytosis process during *A. actinomycetemcomitans* invading HUVEC, the impact of clathrin inhibitors, monodansylcadaverine (MDC) and chlorpromazine (CPZ) were studied in *A. actinomycetemcomitans* adhesion and invasion of HUVEC process. It was found that MDC and CPZ effectively inhibited the PC positive *A. actinomycetemcomitans* invasion of HUVEC to  $41.33 \pm 2.59\%$  and  $43.22 \pm 2.67\%$  of the control (Figure 1), respectively.

However, there was no significant difference between the MDC or CPZ pretreated and nonpretreated groups on adherence (Figure 2), which showed that the MDC and CPZ inhibition of clathrin could not stop *A. actinomycetemcomitans* adhesion to HUVEC.

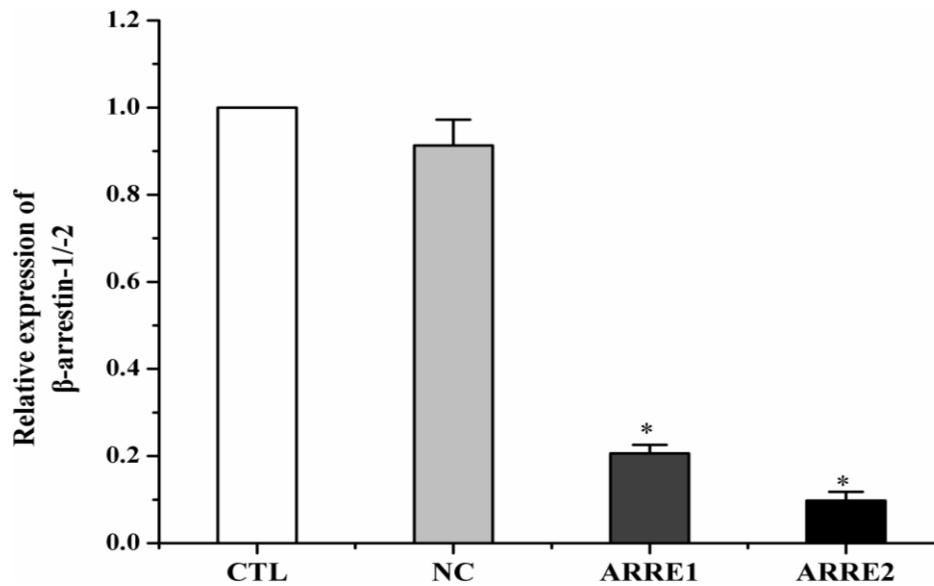
### $\beta$ -Arrestins role in *A. actinomycetemcomitans* internalization by HUVEC

To study the role of  $\beta$ -arrestins in the process of *A. actinomycetemcomitans* adhesion and invasion of HUVEC, double-stranded siRNAs for  $\beta$ -arrestin-1,  $\beta$ -arrestin-2 and control were chemically synthesized and transfected in HUVEC, and then the effects on *A. actinomycetemcomitans* adhesion and invasion of HUVEC were investigated. First, it was detected that intracellular level of  $\beta$ -arrestins was targetedly reduced after transfection when compared with untransfected group and negative control group (Figures 3 and 4). Then the invasion of PC positive *A. actinomycetemcomitans* into HUVEC was tested and it was  $53.12 \pm 2.81$  and  $56.00 \pm 2.37\%$  of the control (Figure 5), respectively. The invasion was effectively blocked by  $\beta$ -arrestins siRNA.

However, there was no significant difference in the number of bacteria adhered to the cells (Figure 6), which indicated that  $\beta$ -arrestins siRNA could not inhibit the PC binding to PAFR on the cell surface.



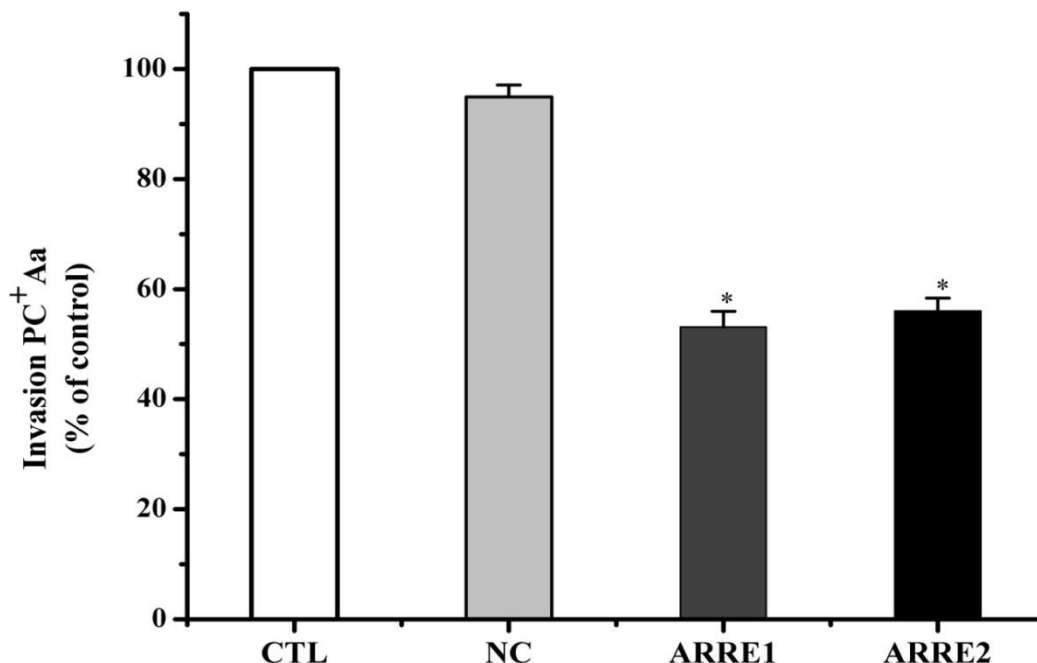
**Figure 3.** Cell transfection of  $\beta$ -arrestin-1/2 siRNA observed under a fluorescence microscope (40 $\times$ ). Transfected cells showed a green fluorescence.



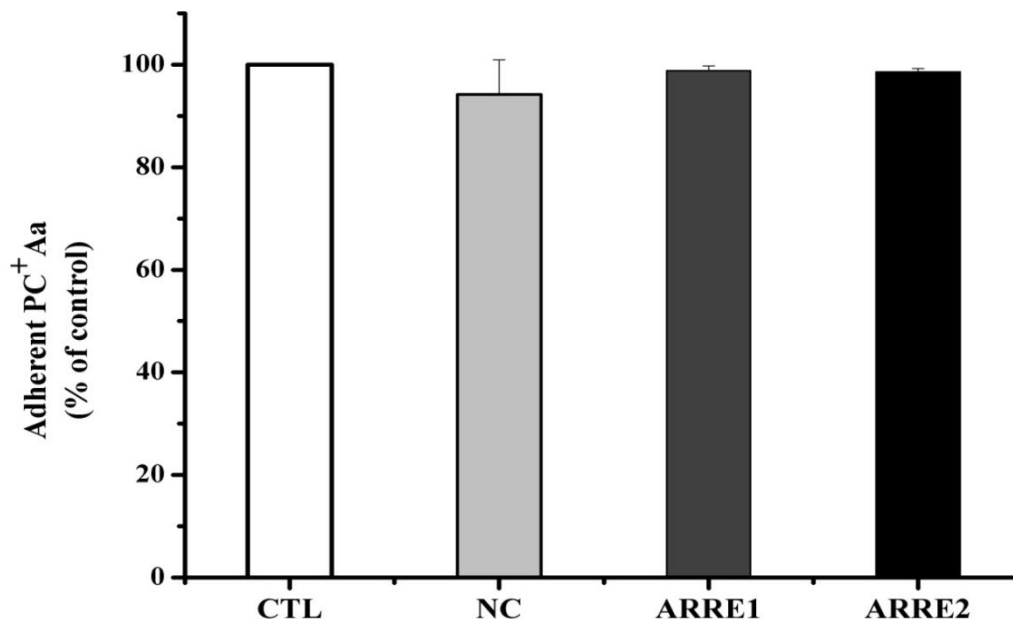
**Figure 4.** Real-time reverse-transcription PCR was used to detect  $\beta$ -arrestin-1 mRNA expression in HUVEC as described in the 'materials and methods' section. Values shown are expressed as percent of level of each siRNA of  $\beta$ -arrestin in control-transfected HUVEC. Error bars indicate standard deviations (\* $P < 0.05$ ).

Besides, we also examined the effect of clathrin and  $\beta$ -arrestins in PC negative *A. actinomycetemcomitans*

groups, there was no significant difference in the adhesion and invasion of HUVEC between PC negative



**Figure 5.**  $\beta$ -Arrestin-1/2 siRNA block PC positive *A. actinomycetemcomitans* invasion of HUVEC. The relative invasion of PC positive *A. actinomycetemcomitans* (%) was examined as described earlier. Data are means  $\pm$  standard deviation (\* $P$ <0.05). CTL: Group without cell transfection; NC: Negative control group.



**Figure 6.** Relative adhesion of PC positive *A. actinomycetemcomitans* (%) after  $\beta$ -arrestin-1/2 siRNA transfected into HUVEC. The adhesion examination was carried out as described before ( $P$ <0.05). The adhesion showed no significant difference between transfected and untransfected groups.

groups (data not shown).

## DISCUSSION

In the present study, we showed new evidences of the

mechanism involved in adherence and invasion in human vascular endothelial cells by *A. actinomycetemcomitans* bearing PC. We demonstrated here that clathrin and  $\beta$ -arrestins participated in *A. actinomycetemcomitans* invasion of host cells.

Previous data have extensively indicated that *A. actinomycetemcomitans* interacts with host cells via a mechanism of specific receptors (Meyer et al., 1997). Schenken and coworkers had reported that PAFR had participated in the invading HUVEC by *A. actinomycetemcomitans* bearing PC (Schenkein et al., 2000; Purkall et al., 2002). Clathrin and  $\beta$ -arrestins have been shown to play important roles in regulating PAFR during bacterial endocytosis. *S. pneumoniae* required the scaffold function of the  $\beta$ -arrestins to recruit clathrin in PAFR-mediated endocytosis by endothelial cells (Radin et al., 2005). Besides, Smani et al. (2012) reported that clathrin and  $\beta$ -arrestin-1 and 2 are involved in *A. baumannii* invasion of lung epithelial cells. In this study, we found that clathrin inhibitors MDC and CPZ significantly inhibited *A. actinomycetemcomitans* bearing PC invasion of HUVEC. Additionally,  $\beta$ -arrestin-1 and -2 siRNA prevented *A. actinomycetemcomitans* internalization of HUVEC. These findings are the first report for clathrin and  $\beta$ -arrestin-1 and -2 that participated in *A. actinomycetemcomitans* invasion of endothelial cells.

## Conclusion

In summary, we proposed that PC-positive *A. actinomycetemcomitans* may invade endothelial cells via a mechanism dependent upon the involvement of clathrin and  $\beta$ -arrestins in mediating the PAFR activation by bacterial PC. However,  $\beta$ -arrestins-mediated receptor internalization can also regulate signal transduction by activating signaling proteins such as ERK1/2, p38 MAPK and JNK (Vroon et al., 2006), which also take part in *A. actinomycetemcomitans* invasion of host cells. Therefore, further studies are needed to demonstrate these other mechanisms involved in  $\beta$ -arrestins-mediated infection by *A. actinomycetemcomitans* bearing PC.

## Conflict of Interests

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

## ACKNOWLEDGEMENT

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## Full Length Research Paper

# Bio-efficacy of fungicides, bioagents and plant extracts/botanicals against *Alternaria carthami*, the causal agent of *Alternaria* blight of Safflower (*Carthamus tinctorius* L.)

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A total of eleven fungicides namely: mancozeb 75 WP, chlorothalonil 75 WP, copper oxychloride 50 WP, thiram 75 WP, captan 50 WP, difenconazole 25 EC, carbendazim 50 WP, hexaconazole 5 EC, propiconazole 25 EC, penconazole 10 EC and metalyxil + mancozeb (Ridomil) 68 WP were evaluated (at 500, 1000 and 1500 ppm each) *in vitro* against *Alternaria carthami*. All the fungicides tested caused significant inhibition at all three concentrations tested over untreated control. The average inhibition recorded with the test fungicides was ranged from 28.39% (chlorothalonil) to 94.44% (carbendazim). However, carbendazim recorded significantly highest average mycelial growth inhibition (94.44%). The second and third best fungicides found were mancozeb (85.43%) and thiram (83.33%). These were followed by hexaconazole (82.95%), ridomil (82.83%) and difenconazole (77.16%). All the six fungal and one bacterial bioagents/antagonists evaluated *in vitro* were found antifungal/antagonistic against *A. carthami*. However, *T. viride* was found most effective and recorded significantly highest mycelial growth inhibition (94.07%) of the test pathogen over untreated control. The second and third best bioagents/antagonists found were *T. hamatum* and *T. koningii* which recorded mycelial growth inhibition 85.18 and 81.11%, respectively. All the eleven plant extract/botanicals were evaluated *in vitro* (each at 10, 15 and 20%) against *A. carthami*. The mean percentage mycelial growth inhibition recorded with all the test botanicals was ranged from 19.26 (*A. racemosus*) to 62.47 (*A. sativum*) per cent. However, *A. sativum* was found most fungistatic which recorded significantly highest mean mycelial growth inhibition (62.47%). The second and third best botanicals found were *D. metal* (49.87%) and *C. longa* (46.91%). Thus, all the botanicals tested were found fungistatic antifungal and caused significant inhibition of *A. carthami* over untreated control.

**Key words:** *Alternaria carthami*, *in vitro*, fungicides, botanicals, bioagents, inhibition.

## INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is one of the important oilseed crops of the world valued for its highly nutritious edible oil. Safflower seed contains about 25-32% oil. It belongs to family compositae and believes to

be native of Afganistan. Safflower is known to suffer from many fungal diseases at different stage of crop growth (Bhale et al., 1998). Leaf spot/blight (*Alternaria carthami*), wilt (*Fusarium oxysporum* f. sp. *carthami*), root rot

(*Rhizoctonia batatiocla*), powdery mildew (*Erysiphe cichoracearum* DC), anthracnose (*Colletotrichum capsici*).

Out of several diseases reported on safflower, *Alternaria* blight caused by *A. carthami* is one of the most important diseases. This disease was first reported by Chowdhury (1944) at Pune in India. In India, disease plays an important role in safflower cultivation and responsible to cause 25 to 60% yield losses every year. In general, the season was suitable for the development of foliar diseases particularly for *Alternaria* leaf spot/ blight development as there were rains after sowing in Maharashtra and a disease severity from 10 to 95% was observed. In Marathwada region of Maharashtra, *Alternaria* leaf spot intensity of 30 to 40%, while in Karnataka and Andhra Pradesh maximum intensity of *Alternaria* leaf spot upto 25 and 20%, respectively

The disease has been reported to cause seed yield losses to the tune of 10-25% (Indi et al., 1988). Under severe conditions, it has been reported to cause 50% loss in yield (Indi et al., 1986). An extensive survey work carried out by Deokar et al. (1991) revealed the predominance of *Alternaria* leaf spot disease on safflower in the traditional safflower growing areas in the scarcity zone of Maharashtra.

The *Alternaria* leaf spot symptoms appears as small brown to dark spots with concentric rings appears first on lower leaves and later spread on upper ones. These spots increases in number and size coalesce and form large irregular lesions. The center of mature spot usually becomes lighter in colour. Infected seed may show dark sunken lesions on the testa at the floret end. Seed may rot or seedling damping off and brown spots up to 5 mm in diameter appear on cotyledons. It has therefore become highly essential to have a good knowledge of such association, method of detection as well as way to control them. Considering economic importance of the disease, the present investigation was undertaken.

## MATERIALS AND METHODS

### *In vitro* efficacy of fungicides

Efficacy of eleven fungicides namely: mancozeb 75 WP, chlorothalonil 75 WP, copper oxychloride 50 WP, thiram 75 WP, captan 50 WP, difenconazole 25 EC, carbendazim 50 WP, hexaconazole 5 EC, propiconazole 25 EC, penconazole 10 EC, metalaxyl + mancozeb (Ridomil) 68 WP were evaluated (500, 1000, 1500 ppm each) *in vitro* against *A. carthami* by Poisoned food technique (Nene and Thapliyal, 1993). Based on active ingredient, the requisite quantity of each fungicide was calculated and mixed thoroughly with autoclaved and cooled (40°C) Potato Dextrose Agar medium (PDA) in conical flasks to obtain desired concentrations of 500, 1000 and 1500 ppm. Plain PDA medium without fungicides served

as untreated control. Fungicide amended PDA medium was then poured aseptically in Petri plates (90 mm dia) and allowed to solidify at room temperature. After solidification of the medium, all the plates were inoculated aseptically with 5 mm culture disc of the test fungus obtained from a week old actively growing pure culture of *A. carthami*. The disc was placed on PDA in inverted position in the centre of the Petri plate and plates were incubated at  $27 \pm 1^\circ\text{C}$ . Each treatment was replicated thrice. Observations on radial mycelial growth/colony diameter were recorded at 24 h interval and continued till the untreated control plate was fully covered with mycelial growth of the test fungus. Per cent mycelial growth inhibition of the test pathogen with the test fungicides over untreated control was calculated by applying the formula given by Vincent (1927).

$$\text{Percent Inhibition (I)} = \frac{C - T}{C} \times 100$$

Where, C = Growth (mm) of test fungus in untreated control plates  
T = Growth (mm) of test fungus in treated plates

### *In vitro* efficacy of bioagents

Six fungal antagonists namely: *Trichoderma viride*, *T. harzianum*, *T. hamatum*, *T. koningii*, *T. lignorum*, *Gliocladium virens* and one bacterial antagonist namely: *Pseudomonas fluorescens* were evaluated *in vitro* against *A. carthami* applying Dual culture technique (Dennis and Webster, 1971). Seven days old cultures of the test bioagents and test fungus (*A. carthami*) grown on (PDA, NA) were used for the study. Discs (5 mm dia) of PDA along with culture growth of the test fungus and bioagents were cut out with sterilized cork borer. Then two culture discs, one each of the test fungus and bioagent were placed at equidistance and exactly opposite with each other on solidified PDA medium in Petri plates under aseptic conditions and plates were incubated at  $27 \pm 1^\circ\text{C}$  PDA. The plates inoculated in centre only with culture disc of test fungus were maintained as untreated control and all the treatments were replicated thrice.

Observations on linear mycelial growth of the test fungus and bioagents were recorded at an interval of 24 h and continued till untreated control plates were fully covered with mycelial growth of the test fungus. Per cent inhibition of the test fungus over untreated control was calculated by applying the formula given by Arora and Upadhyay (1978).

$$\text{Per cent growth inhibition (PI)} = \frac{\text{Colony growth in control plate} - \text{Colony growth in intersecting plate}}{\text{Colony growth in control plate}} \times 100$$

### *In vitro* evaluation of botanicals (plant extracts)

Aqueous extracts of 11 botanicals namely: Mehandi (*Lawsonia innermis*), Ginger (*Zingiber officinale*), Dhatura (*Datura metal*), Tulsi (*Ocimum sanctum*), Parthenium (*Parthenium hysterophorus*), Neem (*Azadirachta indica*), Garlic (*Allium sativum*), Turmeric (*Curcuma longa*), Adulsa (*Adhotoda vasica*), Satawari (*Asparagus recemosus*) and Ghaneri (*Lantana camera*) were evaluated against *A. carthami*, applying poisoned food technique. Leaf extract of the test botanicals were prepared by grinding with mixture-cum grinder. The

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**Table 1.** *In vitro* efficacy of fungicides at different concentrations on mycelial growth and inhibition of *A. carthami*.

Treatment	Mean colony Dia (mm)* at Conc.			Av. Colony Dia. (mm)	% Inhibition at Conc.			Mean % inhibition
	500 ppm	1000 ppm	1500 ppm		500 ppm	1000 ppm	1500 ppm	
T <sub>1</sub> : Mancozeb	17.33	13.00	9.00	13.11	80.73 (53.85)	85.55 (58.82)	90.00 (64.17)	85.43(58.94)
T <sub>2</sub> : Chlorothalonil	68.33	65.33	59.66	64.44	24.07 (13.92)	27.40(15.90)	33.70 (19.69)	28.39 (16.50)
T <sub>3</sub> : Copperoxy chloride	49.66	45.00	39.33	44.66	44.79 (26.62)	49.99 (30.00)	56.29 (34.26)	50.35 (30.29)
T <sub>4</sub> : Thiram	19.66	14.66	10.66	14.99	78.14 (51.40)	83.70 (56.84)	88.15 (61.85)	83.33 (56.69)
T <sub>5</sub> : Captan	46.00	41.33	37.66	41.66	48.88 (29.26)	54.07 (32.73)	58.15 (35.54)	53.70 (32.51)
T <sub>6</sub> : Difenconazole	23.16	20.50	18.00	20.55	74.26 (47.95)	72.22 (50.54)	80.00 (52.07)	77.16 (50.18)
T <sub>7</sub> : Carbendazim	05.00	05.00	5.00	05.00	94.44 (70.79)	94.44 (70.79)	94.44 (70.79)	94.44 (70.79)
T <sub>8</sub> : Hexaconazole	21.00	14.16	10.83	15.33	76.66 (50.07)	84.25 (57.41)	87.96 (61.59)	82.95 (56.35)
T <sub>9</sub> : Propiconazole	24.83	21.83	18.33	21.66	72.40 (46.38)	75.74 (49.23)	79.63 (52.77)	75.92 (49.46)
T <sub>10</sub> : Penconazole	25.16	22.05	18.66	22.10	72.03 (46.08)	75.00 (48.58)	79.26 (52.41)	75.43 (49.02)
T <sub>11</sub> :Ridomil-MZ	19.33	15.01	11.83	15.42	78.51 (51.74)	83.14 (56.25)	86.85 (60.29)	82.83 (56.09)
T <sub>12</sub> : Control (Untreated)	90.00	90.00	90.00	90.00	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)
<b>S.E. ±</b>	<b>1.41</b>	<b>0.67</b>	<b>0.70</b>	<b>0.92</b>	<b>0.74</b>	<b>0.63</b>	<b>0.70</b>	<b>0.69</b>
<b>CD (P=0.05)</b>	<b>4.12</b>	<b>1.95</b>	<b>2.04</b>	<b>2.70</b>	<b>2.17</b>	<b>1.83</b>	<b>2.04</b>	<b>2.01</b>

\*: Means of three replications Figures in parenthesis are arc sine transformed value.

100 g washed leaves of each botanical, ginger rhizome and garlic bulbs (100 g each) were macerated in 100 ml distilled water (w/v) separately and the macerate obtained were filtered through double layered muslin cloth. Each of the filtrate obtained was further filtered through Whatman No. 1 filter paper using funnel and volumetric flasks (100 ml cap.). The final clear extracts/filtrates obtained formed the standard plant extracts of 100% concentration. These were evaluated (at 10, 15 and 20% each) *in vitro* against *A. carthami*, applying poisoned food technique (Nene and Thapliyal, 1993) and using potato dextrose agar (PDA) as basal culture medium.

An appropriate quantity of each plant extract (100%) was separately mixed thoroughly with autoclaved and cooled (40°C) PDA medium in conical flasks (250 ml cap) to obtain desired concentrations (10,15 and 20%). Sterilized and cooled PDA medium amended separately with plant extract was then poured (15 to 20 ml/plate) into sterile glass Petri plates (90 mm dia.) and allowed to solidify at room temperature. Each plant extract and its respective concentrations were replicated thrice. The plates containing PDA without any plant extract were maintained as untreated control. Upon solidification of PDA, all the treatment and control plates were aseptically inoculated by placing in the centre a 5 mm mycelial disc obtained from a week old actively growing pure culture of *A. carthami*. Plates containing plain PDA and inoculated with mycelial disc of test fungus served as untreated control. All these plates were then incubated at 27 ± 1°C temperature for a week or till the untreated control plates were fully covered with mycelial growth of the test fungus.

Observations on radial mycelial growth/colony diameter of the test fungus were recorded treatment wise at 24 h interval and continued till mycelial growth of the test fungus was fully covered in the untreated control plates. Per cent inhibition of mycelial growth over untreated control was calculated by applying the formula given by Vincent (1927).

## RESULTS AND DISCUSSION

### *In vitro* evaluation of fungicides

A total of eleven fungicides namely: mancozeb 75 WP,

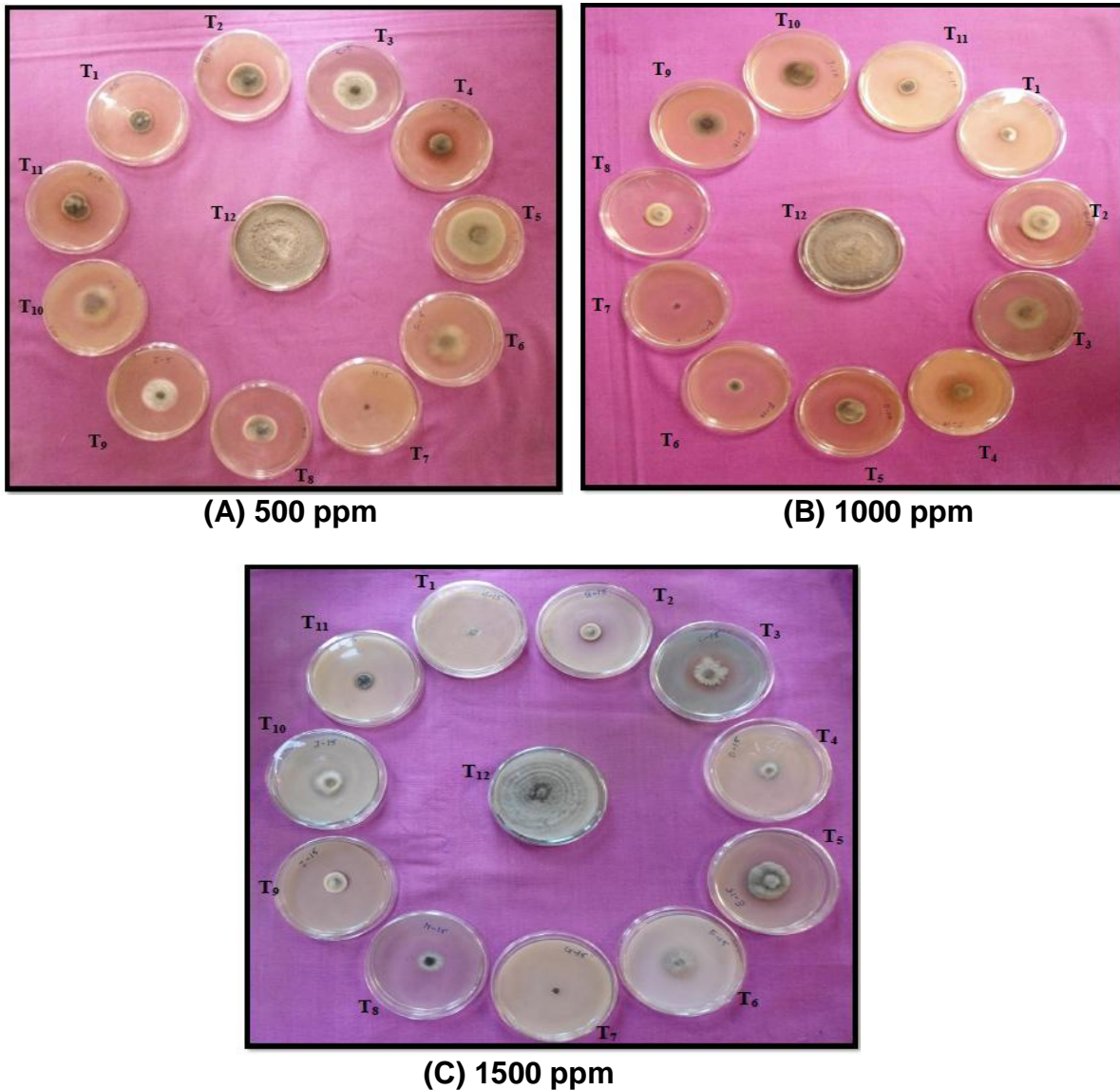
chlorothalonil 75 WP, copper oxychloride 50 WP, thiram 75 WP, captan 50 WP, difenconazole 25 EC, carbendazim 50 WP, hexaconazole 5 EC, propiconazole 25 EC, penconazole 10 EC and metalaxyl + mancozeb (Ridomil) 68 WP were evaluated (at 500, 1000 and 1500 ppm each) *in vitro* against *A. carthami*, applying poisoned food technique and using potato dextrose agar (PDA) as basal medium. Effect of these fungicides on radial mycelial growth and inhibition of the test pathogen over untreated control were recorded and the results obtained are presented in Table 1, Plate-I A B C, and Figures 1, 2 and 3.

### Mycelial growth/colony diameter

Result (Table 1) revealed that all the fungicides tested covered a wide range of radial mycelial growth/colony diameter of the test pathogen, depending upon their concentrations used (Plate I).

At 500 ppm, radial mycelial growth of pathogen was ranged from 5.00 (carbendazim) to 68.33 (chlorothalonil) mm. However, it was the maximum with chlorothalonil (68.33 mm) and this was followed by copper oxychloride (49.66 mm), Captan (46.00 mm), both of which were at par. Significantly least mycelial growth was recorded with carbendazim (5.00 mm) followed by mancozeb (17.33 mm), ridomil (19.33 mm), hexaconazole (21.00 mm), all three of which were at par. difenconazole (23.16 mm), propiconazole (24.83 mm) and penconazole (25.16 mm) all three of which were at par (Plate I (A) and Figure 1).

At 1000 ppm, radial mycelial growth of the test pathogen was ranged from 5.00 mm (carbendazim) to 65.33 mm (chlorothalonil). All the fungicides tasted exhibited similar trends of mycelial growth as that of 500 ppm. However, it was maximum with chlorothalonil (65.33 mm). This was followed by copper oxychloride (45.00



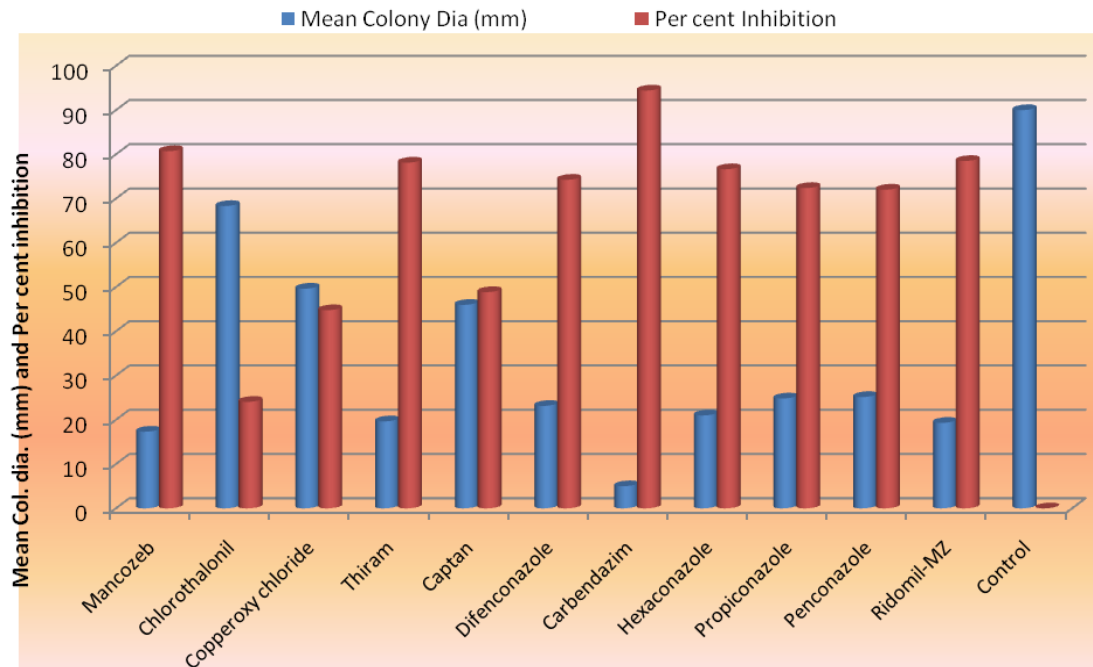
**Plate I.** *In vitro* efficacy of fungicides at 500 ppm (A), 1000 ppm (B) and 1500 ppm concentration (C) on radial mycelial growth and inhibition of *A. carthami*.

mm), Captan (41.33 mm). Significantly least mycelial growth was recorded with the carbendazim (5.00 mm), followed by mancozeb (13.00 mm), hexaconazole (14.16 mm), thiram (14.66 mm), ridomil (15.01 mm) all which were at par and difenconazole (20.50 mm), propiconazole (21.83 mm), penconazole (22.05 mm) all which were at par (Plate I (B) and Figure 2).

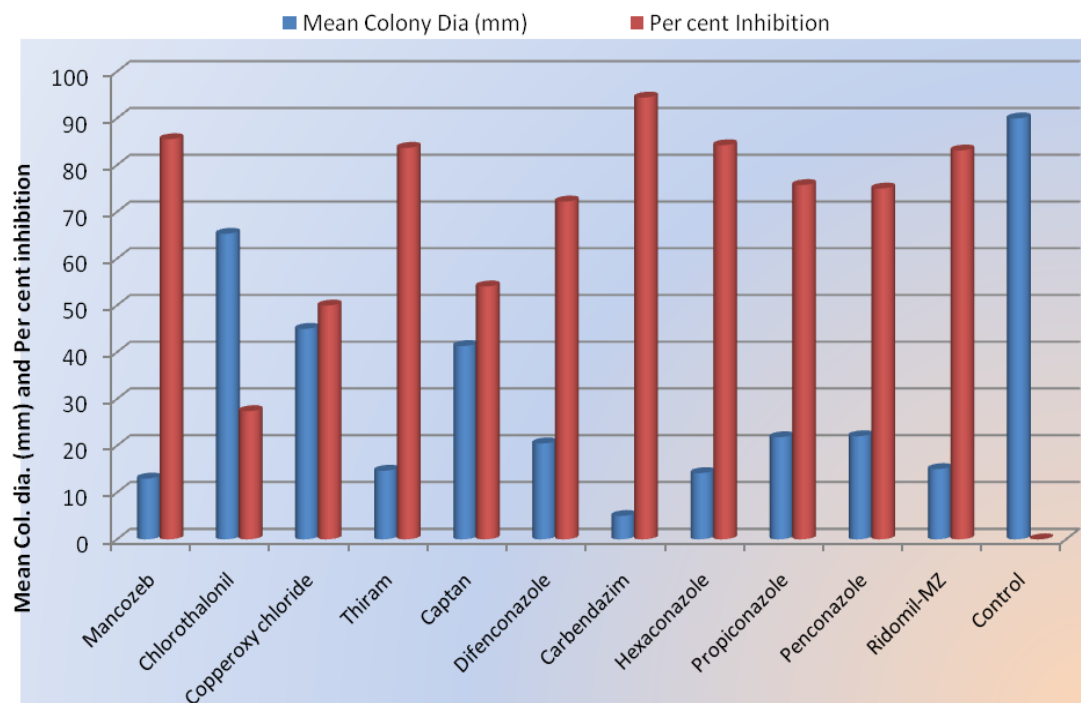
At 1500 ppm, radial mycelial growth of the test pathogen was ranged from 5.00 mm (carbendazim) to 59.66 mm (chlorothalonil). However, it was maximum with chlorothalonil (59.66 mm) and this was followed by copper oxychloride (39.33 mm), captan (37.66 mm) both of which were at par. Significantly minimum mycelial growth was recorded at with the fungicides namely: carbendazim (5.00 mm) followed by mancozeb (9.00 mm), thiram

(10.66 mm), hexaconazole (10.83 mm), ridomil (11.83 mm) all which were at par and difenconazole (18.00 mm), propiconazole (18.33 mm), penconazole (18.66 mm) all which were at par. (Plate I (C) and Figure 3).

Average radial mycelial growth recorded (Table 1) with the fungicides tested (at 500, 1000 and 1500 ppm each) was ranged from 5.00 (carbendazim) to 64.44 mm (chlorothalonil). However, highest mean radial mycelial growth was recorded with chlorothalonil (64.44 mm). This was followed by copper oxychloride (44.66 mm) and captan (41.66 mm). Comparatively minimum average mycelial growth was recorded with the fungicides viz., Carbendazim (5.00 mm), followed by Mancozeb (13.11 mm), Thiram (14.99 mm), Hexaconazole (15.33 mm), Ridomil (15.42 mm), difenconazole (20.55 mm), propiconazole



**Figure 1.** *In vitro* efficacy of fungicide at 500 ppm concentration on mycelia growth and inhibition of *A. carthami*.



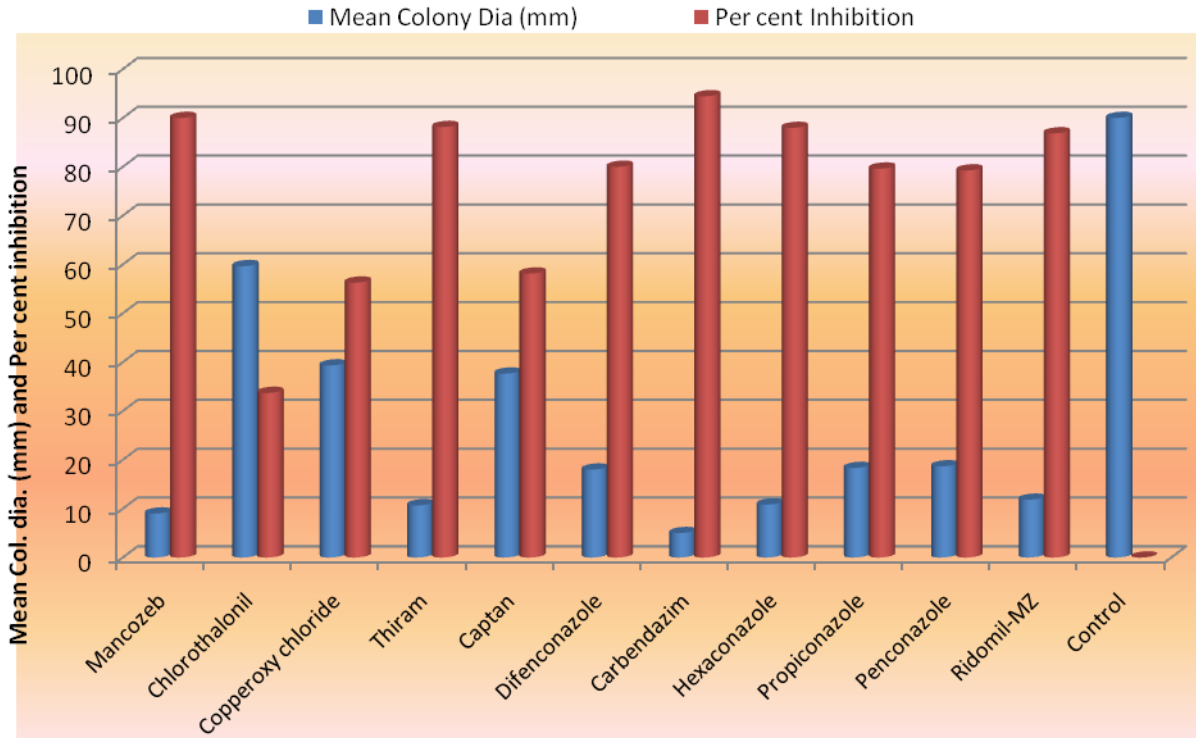
**Figure 2.** *In vitro* efficacy of fungicide at 1000 ppm concentration on mycelia growth and inhibition of *A. carthami*.

(21.66 mm), penconazole (22.10 mm) as compared to 90.00 mm average growth of the pathogen in untreated control.

**Mycelial growth inhibition**

Results (Table 1, Plate I, and Figures 1, 2, 3) revealed





**Figure 3.** *In vitro* efficacy of fungicide at 1500 ppm concentration on mycelia growth and inhibition of *A. carthami*.

that all the fungicides tested (at 500, 1000 and 1500 ppm each) significantly inhibited mycelial growth of the test pathogen over untreated control (0.00%). Further, it was found that percentage mycelial inhibition of test pathogen was increased with increase in concentrations of the fungicides tested.

At 500 ppm, percentage mycelial growth inhibition (Plate I A) was ranged from 24.07 (chlorothalonil) to 94.44 (carbendazim) over untreated control (0.00) per cent. However, significantly highest mycelial growth inhibition was recorded with carbendazim (94.44%). This was followed by mancozeb (80.73%), ridomil (78.51%), thiram (78.14%), hexaconazole (76.66%), difenconazole (74.26%), propiconazole (72.40%) and penconazole (72.03%). The fungicides chlorothalonil, copper oxychloride and captan were found least effective and recorded significantly minimum mycelial inhibition, 24.07, 44.79 and 48.88%, respectively.

At 1000 ppm, percentage of mycelial growth inhibition (Plate I B) was ranged from 27.40% (chlorothalonil) to 94.44% (carbendazim) over untreated control (0.00%). However, significantly highest mycelial growth inhibition was recorded with carbendazim (94.44%). This was followed by fungicides namely: mancozeb (85.5%), hexaconazole (84.25%), thiram (83.70%), ridomil (83.14%), propiconazole (75.74%), penconazole (75.00%) and difenconazole (72.22%) comparatively minimum inhibition was recorded with chlorothalonil (27.40%), copper oxychloride (49.99%) and captan (54.07%).

At 1500 ppm, similar trend of mycelial growth inhibition (Plate I C) with test fungicides was recorded as that of 500 and 1000 ppm and it was ranged from 33.70% (chlorothalonil) to 94.44% (carbendazim) over untreated control (0.00%). However, significantly highest mycelial inhibition was recorded with carbendazim (94.44%). The second and third best fungicides were found mancozeb (90.00%) and thiram (88.15%). This was followed by fungicides namely: hexaconazole (87.96%), ridomil (86.85%), difenconazole (80.00%), propiconazole (79.63%), penconazole (79.26%) and captan (58.15%). Whereas, chlorothalonil and copper oxychloride was found comparatively least effective and recorded minimum inhibition 33.70 and 56.29%, respectively.

Mean percentage mycelial growth inhibition recorded with all fungicides tested (at 500, 1000 and 1500 ppm each) was ranged from 28.39% (chlorothalonil) to 94.44% (carbendazim). However, carbendazim and mancozeb were found most fungistatic each of which recorded significantly highest mean mycelial growth inhibition of 94.44% and 85.43%, respectively over untreated control (0.00%). This was followed by thiram (83.33%), hexaconazole (82.95%), ridomil (82.83%), difenconazole (77.16%), propiconazole (75.92%) and penconazole (75.43%). Less than 51% mean growth inhibition was recorded with chlorothalonil (28.39%) and copper oxychloride (50.35%).

Thus, all the fungicides tested were found fungistatic against the test pathogen *A. carthami* and significantly inhibited its mycelial growth over untreated control.

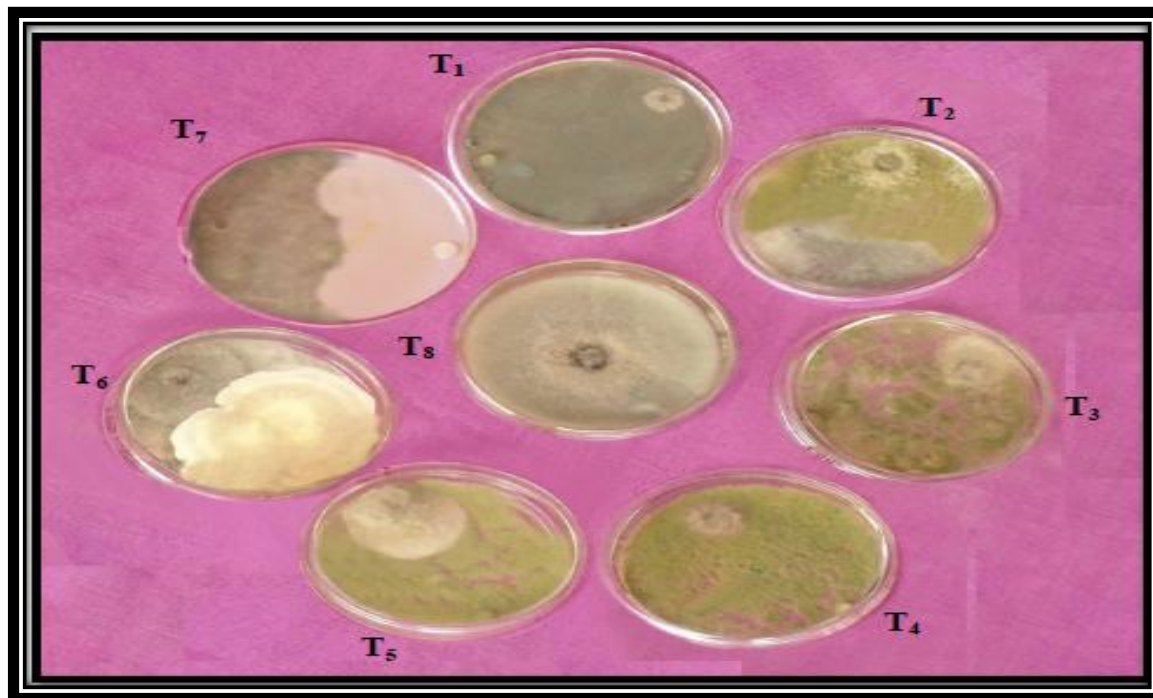


Plate II. *In vitro* efficacy of bioagents on mycelial growth and inhibition of *A. carthami*.

However, carbendazim recorded highest mean mycelial growth inhibition (94.44%), second and third best fungicides were mancozeb (85.83%) and thiram (83.33%). This was followed by hexaconazole (82.95%), ridomil (82.83%), difenconazole (77.16%), propiconazole (75.92%), and penconazole (75.43%). Fungicides chlorothalonil and copper oxychloride were found least effective, which recorded minimum mean mycelial inhibition of 28.39 and 50.35%, respectively. Similar fungistatic effect of the fungicides against *A. carthami* infecting safflower and *Alternaria* spp. infecting many other crops were reported earlier by several workers. (Krishna et al., 1998; Bramhankar et al., 2001; Amaresh and Nargund, 2002; Ambhore et al., 2005; Mesta et al., 2009; Murumkar et al., 2009).

### ***In vitro* evaluation of bioagents**

Six fungal (*Trichoderma viride*, *T. harzianum*, *T. koningii*, *T. hamatum*, *T. lignorum* and *Gliocladium virens*) and one bacterial (*Pseudomonas fluorescens*) bioagents/antagonists were evaluated *in vitro* against *A. carthami*, applying dual culture technique and using PDA as basal medium. The results obtained mycelial growth and inhibition are presented in Plate II, Table 2 and Figure 4.

Results (Table 2) revealed that all the bioagents/antagonists evaluated exhibited fungistatic/antifungal activity and significantly inhibited mycelial growth of *A. carthami* over untreated control (Plate II). Amongst the seven bioagents tested, *T. viride* was found most effective which recorded significantly least linear mycelial growth (5.33 mm) and corresponding highest mycelial growth

inhibition (94.07%) of the test pathogen over untreated control (90.00 mm and 0.00%, respectively). The second and third best bioagents/antagonists found were *T. hamatum* and *T. koningii* which recorded mycelial growth of 13.33 and 17.00 mm, respectively and inhibition of 85.18 and 81.11%, respectively. This was followed by *T. lignorum*, *T. harzianum* and *Gliocladium virens* (growth: 23.33, 34.66 and 41.66 mm, and inhibition: 74.07, 61.48 and 53.71%, respectively). The bacterial antagonists *Pseudomonas fluorescens* was found comparatively least effective and recorded mycelial growth of 45.66 mm and inhibition of 49.26% of the test pathogen.

Thus, all the bioagents evaluated *in vitro* were found fungistatic/antifungal against *A. carthami* and caused significant reduction in mycelial growth of the test pathogen over untreated control. The inhibitory of the *Trichoderma* spp. and *P. fluorescens* against *A. carthami* may be attributed to the mechanisms namely: antibiosis, lysis, mycoparasitism, competition and production of volatile substances.

Results of the present study on antagonistic effects of the *Trichoderma* spp. and *P. fluorescens* against *Alternaria* spp. are in conformity with those reported earlier by several workers (Ghosh et al., 2002; Singh et al., 2005; Mishra and Gupta, 2008).

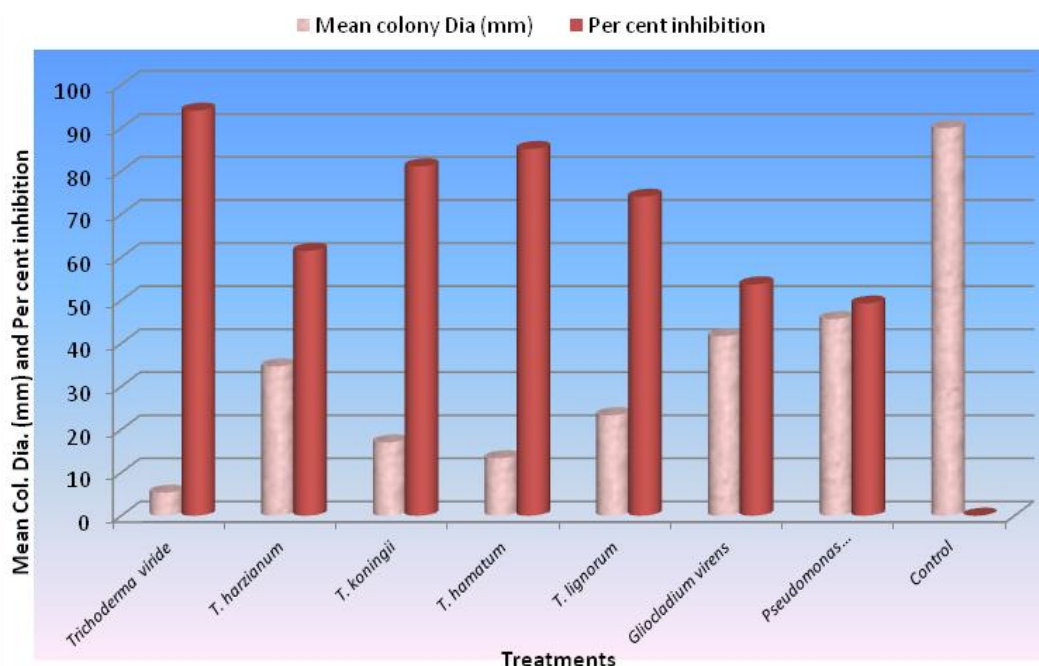
### ***In vitro* efficacy of plant extracts/botanicals**

A total of 11 plant extracts/botanicals namely: Mehandi (*Lawsonia innermis*), Ginger (*Zingiber officinale*), Dhatura (*Datura meta*), Tulsi (*Osmium sanctum*), Parthenium

**Table 2.** *In vitro* efficacy of different bioagents on mycelial growth and inhibition of *A. carthami*.

Treatment	Mean colony Dia(mm)* of pathogen	Inhibition (%)
T <sub>1</sub> : <i>Trichoderma viride</i>	5.33	94.07 (70.17)
T <sub>2</sub> : <i>T. harzianum</i>	34.66	61.48 (37.34)
T <sub>3</sub> : <i>T. koningii</i>	17.00	81.11 (54.43)
T <sub>4</sub> : <i>T. hamatum</i>	13.33	85.18 (58.43)
T <sub>5</sub> : <i>T. lignorum</i>	23.33	74.07 (47.79)
T <sub>6</sub> : <i>Gliocladium virens</i>	41.66	53.71 (32.48)
T <sub>7</sub> : <i>Pseudomonas fluorescens</i>	45.66	49.26 (29.50)
T <sub>8</sub> : Control (Untreated)	90.00	00.00 (00.00)
<b>SE ±</b>	<b>0.84</b>	<b>0.82</b>
<b>C.D. (P = 0.05)</b>	<b>2.51</b>	<b>2.46</b>

\*Mean of three replications. Figures in parenthesis are arc sine transformed value.

**Figure 4.** *In vitro* efficacy of different bioagents on mycelial growth and inhibition of *A. carthami*

(*Parthenium hysterophorus*), Neem (*Azadirachta indica*), Garlic (*Allium sativum*), Turmeric (*Curcuma longa*), Adulsa (*Adhotoda vasica*), Satawari (*Asparagus recemosus*) and Ghaneri (*Lantana camera*) were evaluated (at 10, 15 and 20% each) *in vitro* against *A. carthami*, applying poisoned food technique and using PDA as a basal medium. Results obtained on the effects of the test botanicals/phytoextracts on radial mycelial growth and inhibition of the test pathogen over untreated control were recorded and presented in the Table 3, Plate III A, B, C and Figures 5, 6, 7.

### Radial mycelial growth

Results (Table 3, Plate III) revealed that all the botanicals

/plant extracts tested exhibited a wide range of radial mycelial growth of test pathogen, depending up on their concentration used and it was decreased with increase in concentration of the botanicals tested.

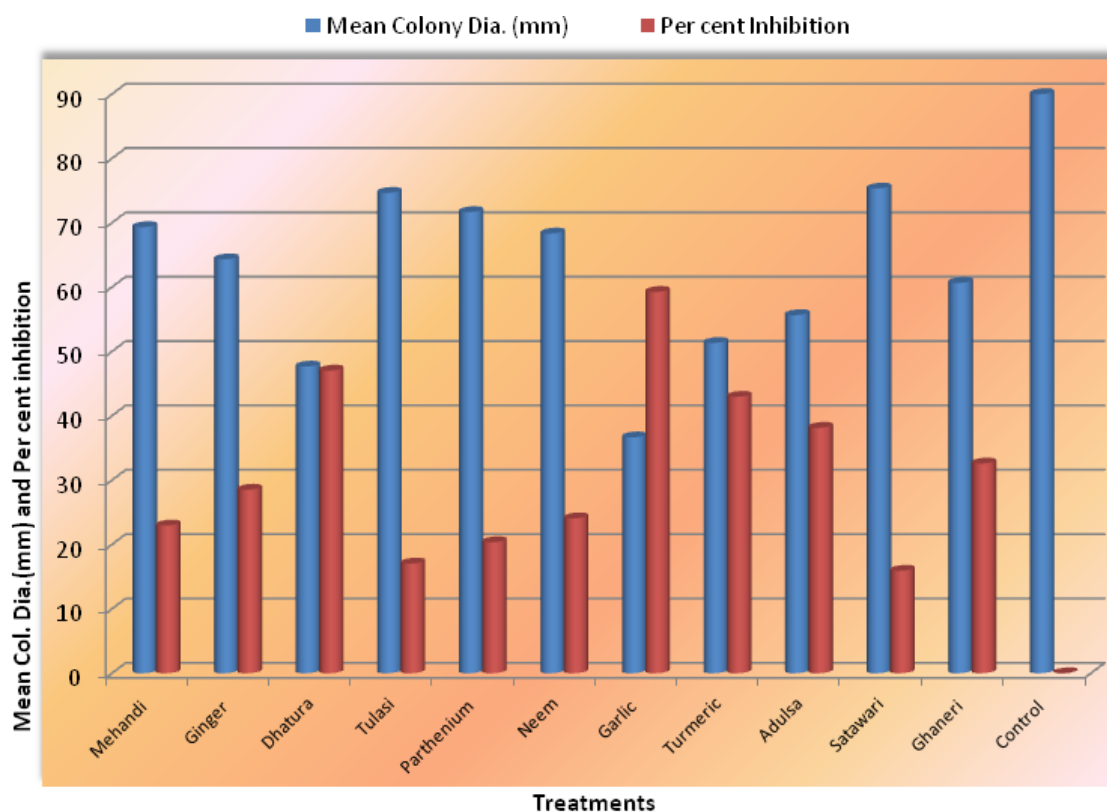
At 10%, radial mycelial growth of the test pathogen was ranged from 36.66 (*A. sativum*) to 75.33 (*A. racemosus*) mm. However, it was maximum with *A. racemosus* (75.33 mm). This was followed by *O. sanctum* (74.66 mm), *P. hysterophorus* (71.66 mm), *L. innermis* (69.33 mm), *A. indica* (68.33 mm), three of which were at par and *Z. officinale* (64.33 mm), *L. camera* (60.66 mm), *A. vasica* (55.66 mm), *C. longa* (51.33 mm) of which were at par. comparatively least mycelial growth was recorded with the botanicals *viz.*, *A. sativum* (36.66 mm) and *D. metal* (47.66 mm) Plate III A, Figure 5.



**Table 3.** *In vitro* efficacy of different plant extracts/botanicals on mycelial growth and inhibition of *A. carthami*.

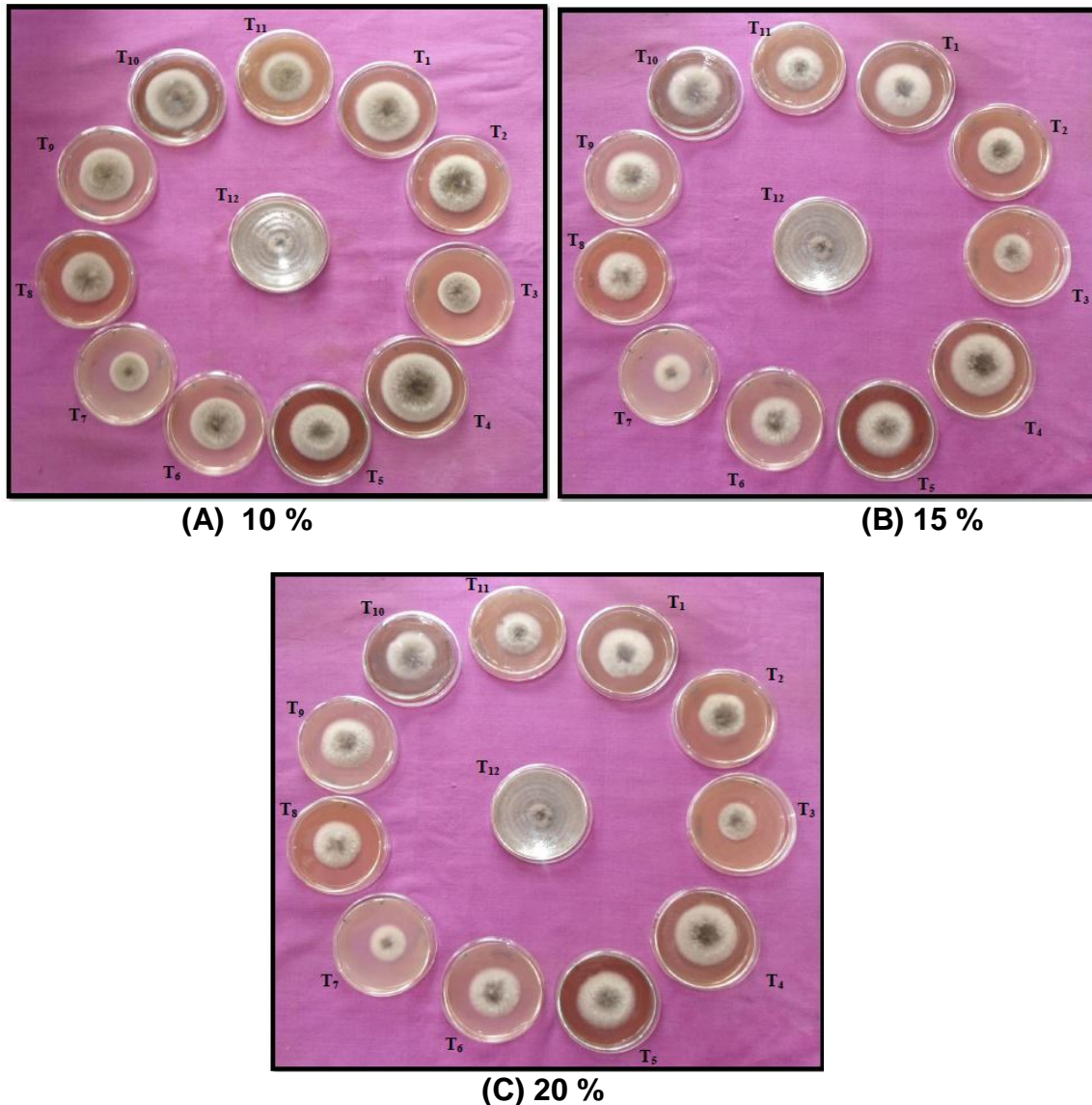
Treatment	Colony Dia. Mean (mm) * at Conc.			Mean (mm)	% Inhibition at Conc.			Mean % Inhibition
	10 %	15 %	20 %		10 %	15 %	20 %	
T <sub>1</sub> : Mehandi ( <i>L. innermis</i> )	69.33	64.66	58.33	64.10	22.96 (13.27)	28.15 (16.34)	35.18 (20.59)	28.76 (16.73)
T <sub>2</sub> : Ginger ( <i>Z. officinale</i> )	64.33	58.33	56.33	59.66	(28.52 (16.56)	35.18 (20.59)	37.41 (21.96)	33.70 (19.70)
T <sub>3</sub> : Dhatura ( <i>D. metal</i> )	47.66	44.66	43.00	45.10	47.04 (28.05)	50.37 (30.24)	52.22 (31.47)	49.87 (29.83)
T <sub>4</sub> : Tulasi ( <i>O. sanctum</i> )	74.66	72.33	70.00	72.33	17.04 (10.01)	19.63 (11.31)	22.22 (12.83)	19.63 (11.38)
T <sub>5</sub> : Parthenium ( <i>P. hysterophorus</i> )	71.66	67.33	63.00	67.33	20.37 (22.53)	25.18 (14.58)	30.00 (17.45)	25.18 (18.18)
T <sub>6</sub> : Neem ( <i>A. indica</i> )	68.33	66.33	61.00	65.22	24.07 (13.92)	26.30 (23.93)	32.22 (18.79)	27.53 (18.88)
T <sub>7</sub> : Garlic ( <i>A. sativum</i> )	36.66	34.00	30.66	33.77	59.26 (35.28)	62.22 (37.66)	65.93 (41.23)	62.47 (38.05)
T <sub>8</sub> : Turmeric ( <i>C. longa</i> )	51.33	47.33	44.66	47.77	42.96 (25.44)	47.41 (28.29)	50.37 (30.24)	46.91 (27.99)
T <sub>9</sub> : Adulsa ( <i>A. vasica</i> )	55.66	53.66	52.00	53.77	38.15 (22.42)	40.37 (23.80)	42.22 (24.97)	40.24 (23.73)
T <sub>10</sub> : Satawari ( <i>A. racemosus</i> )	75.33	73.33	69.00	72.66	15.93 (9.16)	18.52 (10.52)	23.33 (13.49)	19.26 (11.06)
T <sub>11</sub> : Ghaneri ( <i>L. camera</i> )	60.66	57.66	53.33	57.21	32.60 (19.01)	35.93 (21.04)	40.74 (24.03)	36.42 (21.36)
T <sub>12</sub> : Control (Untreated)	90.00	90.00	90.00	90.00	00.00 (00.00)	0.00 (0.00)	00.00 (0.00)	00.00 (00.00)
<b>S.E. ±</b>	<b>0.79</b>	<b>0.63</b>	<b>0.75</b>	<b>0.72</b>	<b>0.87</b>	<b>0.69</b>	<b>0.52</b>	<b>0.69</b>
<b>CD (P=0.05)</b>	<b>2.31</b>	<b>1.83</b>	<b>2.20</b>	<b>2.11</b>	<b>2.61</b>	<b>2.08</b>	<b>1.53</b>	<b>2.07</b>

\*: Means of three replications. Figures in parenthesis are arc sine transformed value.

**Figure 5.** *In vitro* efficacy of 10% plant extracts on mycelial growth and inhibition of *A. carthami*.

At 15 per cent, radial mycelial growth of the test pathogen was ranged from 34.00 (*A. sativum*) to 73.33 (*A. racemosus*) mm. However, it was maximum with *A. racemosus* (73.33 mm). This was followed by *O. sanctum*

(72.33 mm) both of which were at par and *P. hysterophorus* (67.33 mm), *A. indica* (66.33 mm), *L. innermis* (64.66 mm). All of which were at par to each other and *Z. officinale* (58.33 mm), *L. camera* (57.66 mm), *A. vasica* (53.66 mm)



**Plate III.** *In vitro* efficacy of plant extract/botanicals at 10% (A), 15% (B) and 20% (C) on growth and inhibition *A. carthami*.

of which were at par. Significantly least mycelial growth was recorded with the botanicals *A. sativum* (34.00 mm), *D. metal* (44.66 mm) and *C. longa* (47.33 mm) (Plate III B, Figure 6).

At 20%, radial mycelial growth of the test pathogen was ranged from 30.66 mm (*A. sativum*) to 70.00 mm (*O. sanctum*). However, significantly highest mycelial growth was maximum with *O. sanctum* (70.00 mm). This was followed by *A. racemosus* (69.00 mm) both of which were at par and *P. hysterothorus* (63.00 mm), *A. indica* (61.00 mm), *L. innermis* (58.33 mm), *Z. officinale* (56.33 mm). All of which were at par to each other and *L. camera* (53.33 mm), *A. vasica* (52.00 mm), both of which were at par. Less than 50 mm growth was recorded with the botanicals *C. longa* (44.66 mm) and *D. metal* (43.00 mm)

both of which were at par. Significantly least mycelial growth was recorded with the botanical *A. sativum* (30.66 mm), as compared to untreated control was 90.00 mm (Plate III C, Figure 7).

The mean percentage radial mycelial growth recorded with the plant extracts tested (at 10, 15 and 20% each) was ranged from 33.77 mm (*A. sativum*) to 72.66 mm (*A. racemosus*). However, significantly highest mean mycelial growth was recorded with *A. racemosus* (72.66 mm). This was followed by *O. sanctum* (72.33 mm) both of which were at par and *P. hysterothorus* (67.33 mm), *A. indica* (65.22 mm), *L. innermis* (64.10 mm), *Z. officinale* (59.66 mm), *L. camera* (57.21 mm). Comparatively less mycelial growth was recorded with the botanicals *A. vasica* (53.77 mm), *C. longa* (47.77 mm) and *D. metal*

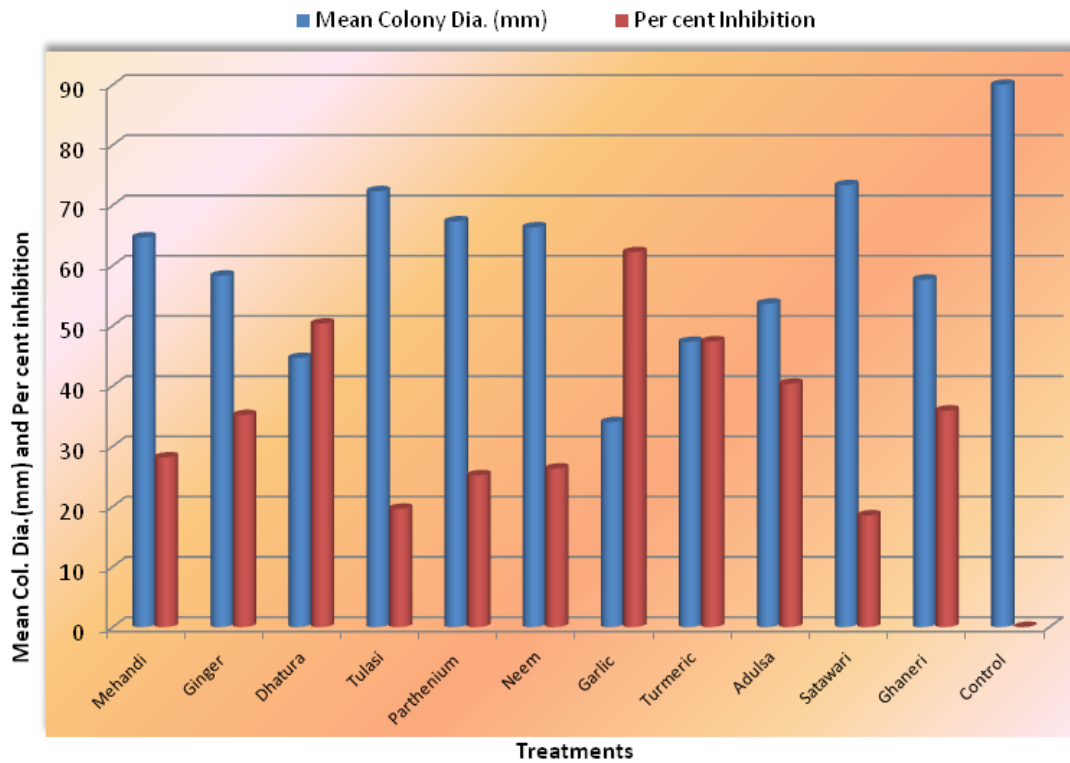


Figure 6. *In vitro* efficacy of 15% plant extracts on mycelial growth and inhibition of *A.carthami*.

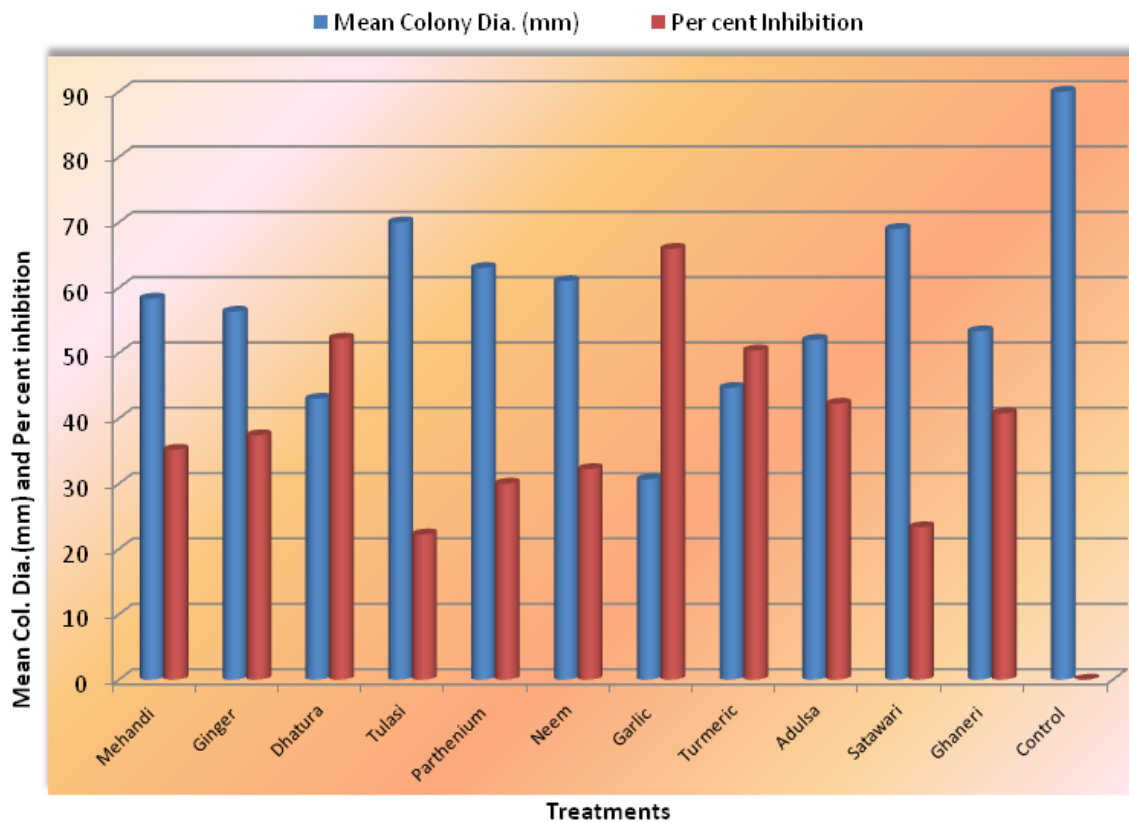


Figure 7. *In vitro* efficacy of 20% plant extracts on mycelial growth and inhibition of *A.carthami*.

(45.10 mm). Significantly least mean mycelial growth was recorded with *A. sativum* (33.77 mm). This was followed by *D. metal* (45.10 mm). The mean mycelial growth was recorded in untreated control was 90.00 mm.

### Mycelial growth inhibition

Results (Table 3, Plate III, A, B, C, and Figures 5, 6, 7) revealed that all the plant extracts tested (at 10, 15 and 20% each) significantly inhibited mycelial growth of the test pathogen over untreated control (0.00%). Further, it was found that per cent mycelial growth inhibition of the test pathogen was increased with increase in concentration of the botanicals tested.

At 10%, mycelial growth inhibition was ranged from 15.93 (*A. racemosus*) to 59.26 (*A. sativum*) per cent. However, significantly highest mycelial growth inhibition was recorded with *A. sativum* (59.26%). This was followed by *D. metal* (47.04%), *C. longa* (42.96%), *A. vasica* (38.15%) and *L. camera* (32.60%). Comparatively less mycelial growth inhibition was recorded with *Z. officinale* (28.52%), *A. indica* (24.07%) and *L. innermis* (22.96%) both of which were at par. Significantly least mycelial growth inhibition was recorded with botanicals namely: *A. racemosus* (15.93%). This was followed by *O. sanctum* (17.04%) and *P. hysterothorus* (20.37%) over untreated control (0.00%).

At 15%, mycelial growth inhibition was ranged from 18.52 (*A. racemosus*) to 62.22 (*A. sativum*) per cent. However, significantly highest mycelial growth inhibition was recorded with *A. sativum* (62.22%). This was followed by *D. metal* (50.37%), *C. longa* (47.41%) and *A. vasica* (40.37%). Comparatively less mycelial growth inhibition was recorded with *L. camera* (35.93%) and *Z. officinale* (35.18%) both of which were at par and *L. innermis* (28.15%), *P. hysterothorus* (25.18%) and *O. sanctum* (19.63%). Significantly least mycelial growth inhibition was recorded with *A. racemosus* (18.52%) over untreated control (0.00%).

At 20%, mycelial growth inhibition was ranged from 22.22 (*O. sanctum*) to 65.93 (*A. sativum*) per cent. However, significantly highest mycelial growth inhibition was recorded with *A. sativum* (65.93%). This was followed by *D. metal* (52.22%), *C. longa* (50.37%) both of which were at par and *A. vasica* (42.22%), *L. camera* (40.74%) both of which were at par. Comparatively less mycelial growth inhibition was recorded with *Z. officinale* (37.41%), *L. innermis* (35.18%), *A. indica* (32.22%) and *P. hysterothorus* (30.00%). Significantly least mycelial growth inhibition was recorded with *A. racemosus* (23.33%) and *O. sanctum* (22.22%) over untreated control (0.00%).

Mean percentage mycelial growth inhibition recorded with all the test botanicals was ranged from 19.26 (*A. racemosus*) to 62.47 (*A. sativum*) per cent. However, *A. sativum* was found most fungistatic and recorded significantly highest mean mycelial growth inhibition (62.47%). The second and third best plant extracts found were *D.*

*metal* (49.87%) and *C. longa* (46.91%). This was followed by *A. vasica* (40.24%), *L. camera* (36.42%), *Z. officinale* (33.70%) and *L. innermis* (28.76%). Comparatively minimum mycelial inhibition was recorded with *A. indica* (27.53%), *P. hysterothorus* (25.18%), *O. sanctum* (19.63%) and *A. racemosus* (19.26%) over untreated control (0.00%).

Thus, all the plant extracts tested were found fungistatic against *A. carthami* and significantly inhibited its mycelial growth over untreated control. However, *A. sativum* recorded highest mean mycelial growth inhibition (62.47%) and this was followed by *D. metal* (49.87%) and *C. longa* (46.91%).

Thus, all the botanicals/plant extracts evaluated *in vitro* were found fungistatic and significantly inhibited mycelial growth of *A. carthami*. The fungistatic effects of the botanicals might be due to the presence of specific antifungal chemical compounds like phenols, tannins, alkaloids, resinous and non volatile substances.

Similar results of antifungal/fungistatic effect of botanicals/plant extracts against *A. carthami* and *Alternaria* spp. were reported earlier by several workers (Singh and Mujumdar, 2001; Ghosh et al., 2002; Shinde et al., 2008; Mesta et al., 2009; Ranaware et al., 2010).

### Conflict of Interests

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

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## Full Length Research Paper

## Assessment of ants as bacterial vector in houses

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Studies of arthropods have fundamental importance to identifying both ant species and microorganisms they carry. The objective of this study was to identify ant morph species found in residences, characterize the bacteria associated with the ants, assess bacterial resistance to antibiotics and analyze the plasmid profiles of the microorganisms found. The ant collections were carried out in the kitchens of 50 residences in the daytime. The bacteria were quantified and the samples were confirmed for the presence of plasmids. The data demonstrate that *Pheidole* sp. (74%) was the most frequent ant among those isolated; the microbiology analysis showed that the genus *Staphylococcus* sp. (90%) was the most prevalent bacterial genus found on ants. This research presented a low frequency of *Klebsiella* sp. (2%), *Enterococcus* sp. (2%) and *Vibrio cholera* (2%). The ants classified as *Pheidole* sp. and *Paratrechina* sp. showed greater presence of microorganisms in the ants' cuticle, and *Pheidole* sp. was prevalent in relation to other ant species. Resistance was found to the antibiotics ampicillin, erythromycin and penicillin. Since there was no plasmid in the samples, it can be suggested that this tolerance is of chromosomal bacterial origin.

**Key words:** Antimicrobial, ants, contamination vector, resistance.

### INTRODUCTION

Of Brazil's 2,000 ant species, 20 to 30 are considered urban nuisances (Campos-Farinha, 2005). When humans seek comfort, they favor the survival of the urban ants offering food, shelter and moisture (Bueno et al., 1999). Most urban ants are omnivorous and present great mobility while looking for food, crossing garbage bins, drains and home environments, promoting contamination of their tegument while enlarging their potential vectorial (Thyssen et al., 2004). While colonizing domestic environments, these arthropods can damage electric appliances and cause contamination and disturbances for

humans (Rodvalho et al., 2007).

Ants have several mutualistic and parasitic relationships with fungus, bacteria, animals and plants (Boursaux-Eude and Gross, 2000). Several studies conducted in hospitals have proven fungus and bacteria presence on the ants' tegument (Pesquero et al., 2008). As they are antibiotic-resistant microorganisms, these insects act like mechanical disease vectors, contributing to nosocomial infection (Moreira et al., 2005). However, some microorganisms are known to be bacteria carried by ants in residences and food establishments (Zarzueta et al.,

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**Table 1.** Kind of bacteria have been reported in ant morphospecies.

Ant	Bacteria
<i>Paratrechina</i> sp.	<i>Streptococcus</i> sp., <i>Staphylococcus</i> sp., <i>Salmonella</i> sp., <i>Vibrio</i> sp., <i>Vibrio cholerae</i> , <i>Bacillus</i> sp., <i>Micrococcus</i> sp. and <i>Enterococcus</i> sp.
<i>Pheidole</i> sp.	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp., <i>Streptococcus</i> sp., <i>Salmonella</i> sp., <i>Micrococcus</i> sp., <i>Klebsiella</i> sp., <i>Vibrio</i> sp., <i>E. coli</i> , <i>Neisseria</i> sp. and <i>Enterobacteria</i> sp.
<i>Brachymyrmex</i> sp.	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp., <i>Neisseria</i> sp. and <i>Vibrio</i> sp.
<i>Dorymyrmex</i> sp.	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp. and <i>Vibrio</i> sp.
<i>Tapinoma melanocephalus</i> sp.	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp. and <i>Enterobacteria</i> sp.
<i>Tetramorium</i> sp.	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp. and <i>Salmonella</i> sp.

2004).

Diseases transmitted by foods (DTA) present a public health problem all over the world, being responsible for high economic and social costs (Welker et al., 2010). DTA thus leads to high mortality and morbidity rates (Martins et al., 2007). Bacteria function has been proven in food contamination cases (Andrade et al., 2003), showing that the microorganisms can be transported by ants.

Antibiotic-resistant bacteria have been studied frequently in urban ants (Kumar, 2012). The indiscriminate use of antibiotics by humans and for animal raising in the 1970's significantly increased the number and types of microorganisms resistant to these drugs (WHO, 2011). This higher resistance to antibiotics constitutes a global concern (van der Donk et al., 2012). Resistance is considered a natural phenomenon and resistance mechanisms are preexistent or naturally modified, both in gene transfers and in changes in bacterial chromosomes or plasmids (García et al., 2003).

The objective of the present work was to identify ant morph species found in residences. These were characterized as bacteria associated with ants. Bacterial resistance to antibiotics was measured and the microorganisms' plasmidial profiles were analyzed.

## MATERIALS AND METHODS

### Study location

The ant collections were done in the city of Morrinhos, Goiás, Brazil (17° 30' 05" a 18° 06' 11" S e 48° 48' 49" a 49° 27' 42" W) in 50 residences in 20 urban neighborhoods.

### Data collection: Mimercofauna

The ants were collected in kitchens in the morning using sugary bait (honey) and protein bait (sausage). The bait was left for an hour, which is approximately the period in which ant trails form. The ants were collected and stored in 70% alcohol with information about the place, date and time of collection.

### Data collection: Microbiological

For microbiological analysis, the insects were collected using sterilized tweezers and cotton before coming into contact with the bait and then stored in new plastic bags, which were labeled and kept in a thermal box at approximately 10°C. After collection, the materials were taken to the laboratory for bacteria cultivation. An ant specimen was immersed in peptone water for 15 min, maintaining the nutritive medium at 37°C in a bacteriological incubator for 24 h.

### Ant morphospecies identification

The collected specimens were identified by genus and morphospecies and, when possible, by species, using a dichotomous key for genus (Fernández, 2003) (Table 1).

### Microbiological analyses

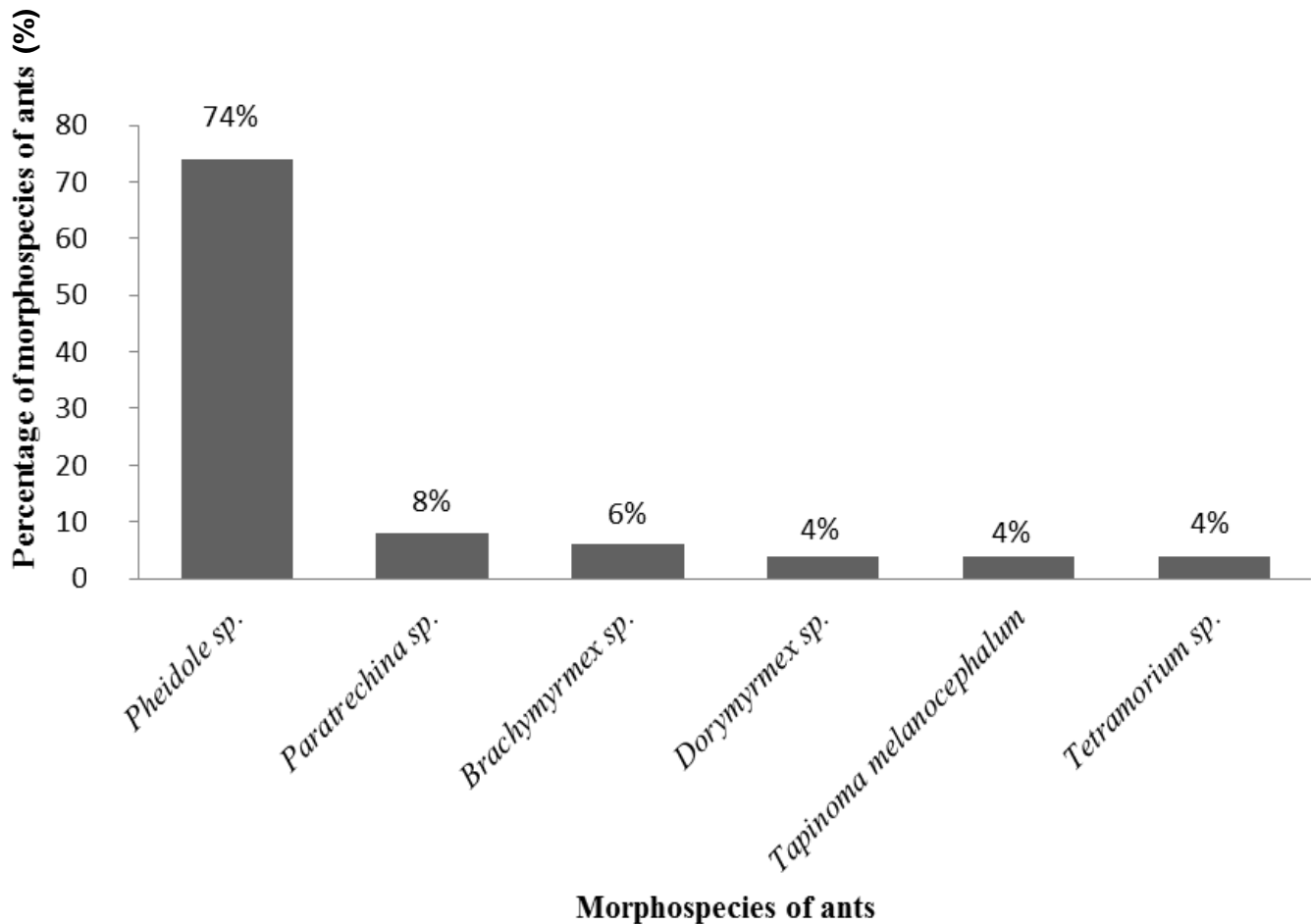
The number of microorganisms was determined by the serial dilution method (undiluted sample and serial dilutions of 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup>) in "Plate Count Agar" (PCA). For bacterial identification, Gram staining was done and the following biochemical tests were performed: catalase, motility, glucose, methyl red, Vogues Proskauer, phenylalanine deaminase, indole, sulfuric acid, urease and Simmons citrate agar. The following enriched mediums were used for identification: MacConkey agar, mannitol salt agar, bright green and EC broth. The results were interpreted according to Bergey's Manual of Systematic Bacteriology.

### Antibiotic resistance analysis

The bacteria samples were seeded by scattering them in a petri dish containing Mueller Hinton agar and the inhibition zone of each paper disc was measured according to the Bauer-Kirby disc diffusion method. An antibiogram was performed to check for resistance to penicillin (10 µg), ampicillin (10 µg), erythromycin (15 µg) and gentamicin (10 µg), which are first- to third-generation antibiotics. The results were classified as sensitive, intermediate and resistant to the antibiotics tested, following the norms established by the National Committee for Clinical Laboratory Standards.

### Data analysis

The antibiogram results were analyzed using the  $\chi^2$  test according



**Figure 1.** Percentage of ant morphospecies found in residence kitchens.

to the Systat 13 program.

#### **Plasmid extraction**

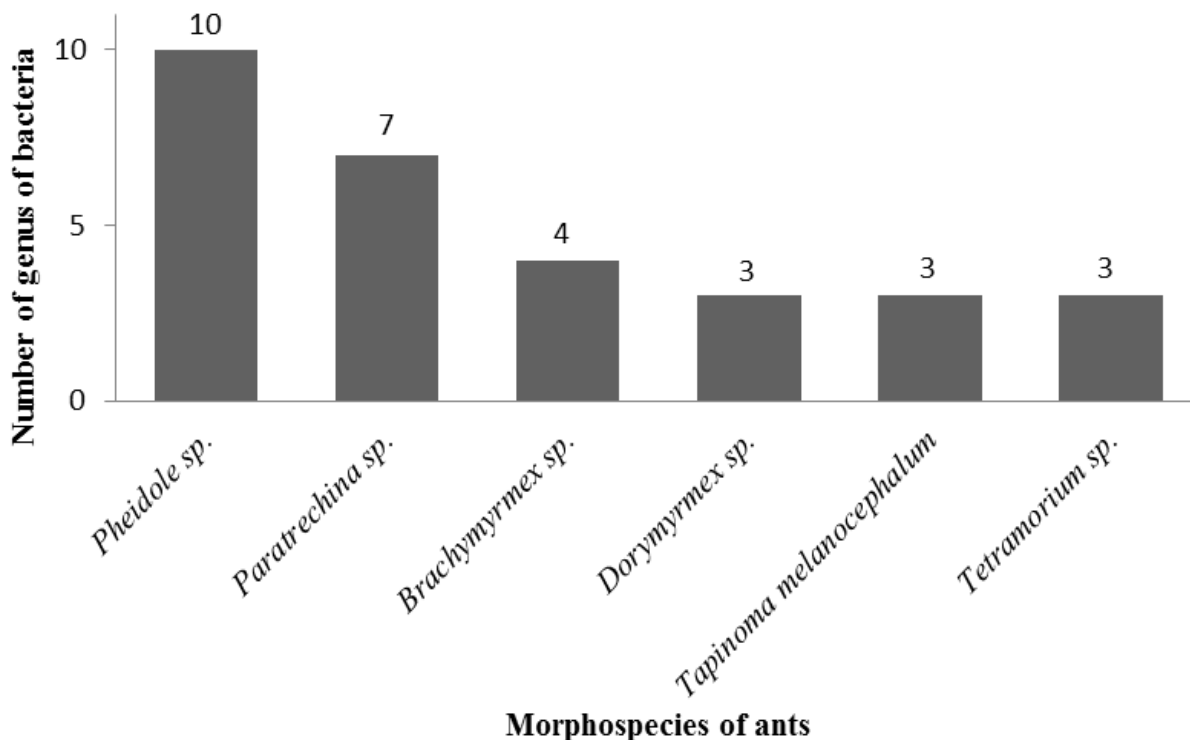
The presence of plasmid DNA was checked with the Pharmacia® FLEXIPREP extraction kit. The procedure was performed according to Sambrook, Fritsh and Maniats (1989). The sample was spiked in peptone water and incubated at 37°C for 24 h. An aliquot of 1.5ml was centrifuged at 5,000xg in eppendorf tubes for 30 s. This process was repeated three times. The sediment was resuspended in 200 µL of solution I and then homogenized. Subsequently, 200 µL of solution II was added and the material was mixed by inverting the tube for 5 min. After that, 200 µL of solution III was added and gently homogenized by inversion for 5 min and centrifuged at 5,000 xg for 5 min (according to the manufacturer's instructions). The sediment corresponding to the chromosomal DNA was discarded and the supernatant containing plasmid DNA was transferred to another tube. The DNA was precipitated by adding 420 µL of isopropanol and, after being homogenized, was placed to rest at room temperature for 10 min. It was then immediately centrifuged at 5,000 × g for 10 min. The supernatant was discarded and the precipitate was left to dry at room temperature. The precipitate was then resuspended in 50 µL of Milli Q water, homogenized and viewed in 1% (w/v) agarose gel with ethidium bromide and analyzed using electrophoresis.

## **RESULTS AND DISCUSSION**

The ants classified as *Pheidole* sp. (74%) constituted the most frequent morph species found. *Dorymyrmex* sp. (4%), *Tapinoma melanocephalum* (4%), and *Tetramorium* sp. (4%), were the least frequent species found (Figure 1). Six ant morph species were found. This can be explained by the fact that these houses do not have the resources necessary for many arthropods to survive (Iop et al., 2009). The ant groups found in this study are common in urban environments (Lutinski et al., 2013). According to Wilson (2003), the genus *Pheidole* is abundant in neotropical regions, its dominance is due to its ability to communication and increased recruitment and aggressiveness in defense of food resources.

When ants and isolated bacteria were correlated, it was observed that the 50 samples collected were contaminated. Due to the *Pheidole* sp. species' abundance, it is responsible for the highest contamination rate. Ten bacterial genus were isolated from *Pheidole* sp. (Figure 2), followed by seven *Paratrechina* sp. isolated genus, and adding the 12 different bacterial\_types found





**Figure 2.** Relative value of bacterial genus isolated per ant.

in the ants.

The quantification was done using the most probable number methodology. The samples were immersed in "Plate Count agar (PCA) and 85.5% of the samples showed CFU/mL (colony forming units) countless count. Residences that showed smaller CFU/mL had been cleaned minutes before collection. It was observed that the houses that were cleaned before the ants were collected showed lower bacterial colonization, corroborating data showing the bactericidal effect of household disinfectants (INMETRO, 2008). This therefore demonstrates that contamination risk due to bacteria can be reduced by using hygienic practices. After the quantification was carried out, the identification of bacteria isolated on the ant tegument was performed. *Staphylococcus* sp. (90%), was the most common bacteria isolated and the *Klebsiella* sp., *Enterococcus* sp. and *Vibrio cholerae* families were the rarest, all having a 2% occurrence (Figure 3). Most of the ants hosted more than one bacterial genus.

Frequency of the *Staphylococcus* genus (90%), can lead to high levels of contamination and cause diseases related to the presence of domestic animals such as dogs and cats that harbor this bacterium in their fur (Hirsh and Zee, 2003). *Staphylococcus* sp. can also contaminate food scraps, causing several food-borne infections (Szweda et al., 2012). This bacteria genus is considered antimicrobial-resistant, being one of the effects of

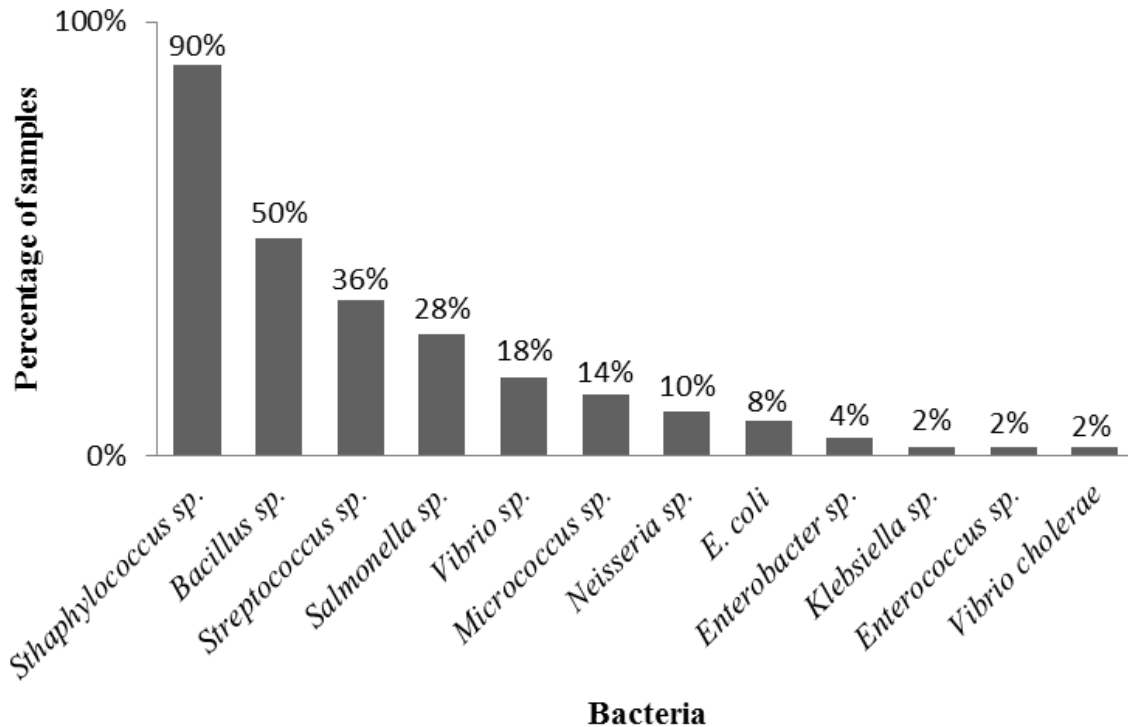
indiscriminate antibiotics use, both by humans and for domesticated animals (Soares et al., 2008).

The Table 2 presents the antibiogram results and demonstrates that all microorganisms tested were gentamicin-susceptible. The results for penicillin, were resistant (54%) and intermediate (44%) ( $\chi^2 = 0.32$ ,  $p = 0.57$ ). Only 2% of the isolated bacteria showed sensitivity. For ampicillin, 74% of the samples tested showed resistance ( $\chi^2 = 35.65$ ,  $p < 0.0001$ ) and erythromycin resistance was 84% ( $\chi^2 = 55.70$ ,  $p < 0.00001$ ) samples. It is important note that the resistances detected in this study involve current antibiotics in clinical therapy. A resistance profile has been observed in household insects (Zarzueta et al., 2004), demonstrating the need for conscious antibiotics use.

The 50 samples were analyzed and no hosted plasmids were found. Any microorganism-hosted plasmid can suggest that antibiotics resistance is due to chromosomal DNA since changes in chromosomal DNA allow bacteria to survive in unfavorable conditions and multiply in addition to conferring antibiotic tolerance (Aly et al., 2012).

#### ACKNOWLEDGEMENTS

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**Figure 3.** Percentage of bacteria types found in isolated ant species.

**Table 2.** Sensitivity and resistance profile of bacteria according to antibiotics tested.

Antibiotic	Resistant number	Intermediate number	Sensitive number
Ampicillin	37	13	0
Erythromycin	3	5	42
Gentamicin	0	0	50
Penicillin	27	22	1

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Full Length Research Paper

# Isolation and identification of probiotic lactic acid bacteria from curd and *in vitro* evaluation of its growth inhibition activities against pathogenic bacteria

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The aim of this study was to isolate and identify probiotic lactic acid bacteria from curd and evaluate *in vitro*, its growth inhibition activities against pathogenic bacteria. A total of nine strains of *Lactobacillus* were isolated from curd and identification of strains was done by biochemical and physiological tests and *Lactobacillus leichmannii*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus coagulans*, *Lactobacillus acidophilus*, *Lactobacillus lactis* and *Lactobacillus rhamnosus* strains were identified from curd. *Lactobacillus* strains survival were also assessed under conditions simulating human GI tract. Therefore, resistance to antibiotics, resistance to low pH, resistance to bile salt and bile salt hydrolysis was performed. Results showed that all tested isolates were able to grow at low pH 3.0, and at 0.3% bile concentration. *L. casei*, *L. delbrueckii* and *L. brevis* showed more resistance to antibiotics. According to haemolytic activity, all examined strains did not exhibit  $\beta$ -haemolytic activity when grown in Columbia human blood agar. Regarding the bile salt hydrolysis, *L. casei* and *L. delbrueckii* showed partial bile salt hydrolysis activity and colony morphology was recorded as differentiated in comparison with the control MRS agar plates. Finally, antimicrobial activities of lactobacillus isolates were tested against five pathogenic bacteria (*Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E. coli* sp.) at pH 6.5 by disc diffusion method. All the tested isolates showed *in vitro* inhibitory zone against pathogenic bacteria. *L. casei* and *L. delbrueckii* showed maximum inhibition zone. In conclusion, the present study showed that *L. casei* and *L. delbrueckii* can be used as potential probiotic lactic acid bacteria.

**Key words:** *Lactobacillus*, curd, probiotics, antibiotic resistance, resistance to low pH, resistance to bile, pathogenic bacteria.

## INTRODUCTION

The term probiotic, literally meaning “for life”, was first addressed by Lilly and Stillwell (1965) and was used to describe substances produced by protozoa to stimulate

the growth of other organisms. Nowadays, the term refers to viable, nonpathogenic microorganisms (bacteria or yeasts) nonpathogenic microorganisms (bacteria or

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yeasts) that, when ingested, are able to reach the intestines in sufficient numbers to confer health benefits to the host (Schrezenmeir and De Vrese, 2001). Commonly used bacterial probiotics include various species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, as well as *Lactococcus lactis* and some *Enterococcus* species (Morrow et al., 2012). It is well established that probiotics confer a number of beneficial health effects to humans and animals. Intake of probiotics stimulates the growth of beneficial microorganisms and reduces the amount of pathogens, thus improving the intestinal microbial balance of the host and lowering the risk of gastro-intestinal diseases (Fuller, 1989; Cross, 2002; Chiang and Pan, 2012). Their benefits also include the alleviation of certain intolerances (such as lactose intolerance), the enhancement of nutrients bioavailability, and prevention or reduction of the prevalence of allergies in susceptible individuals (Isolauri, 2001; Chiang and Pan, 2012). Probiotics are reported to also have antimutagenic, anticarcinogenic, hypocholesterolemic, antihypertensive, anti-osteoporosis and immuno-modulatory effects (Chiang and Pan, 2012). They relieve the symptoms of inflammatory bowel diseases, irritable bowel syndrome, colitis, alcoholic liver disease, constipation and reduce the risk for colon, liver and breast cancers (Prado et al., 2008).

Lactic acid bacteria are Gram positive organisms which are safely applied in medical and veterinary functions (Holzapfel and Schillinger, 2002). Isolation and screening of lactic acid bacteria from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes and the proper selection and balance of lactic acid bacteria used for starter culture is critical for the manufacture of dairy fermented food products with their desirable texture and flavor (Sanders, 2000). Mankind exploited these bacteria for thousands of years for the production of traditionally fermented dairy products because of ability to produce desirable changes in taste, flavor, texture and extending the shelf life of food products (Dave and Shah, 1996). Many studies have reported that the best matrices to deliver probiotic lactic acid bacteria are dairy fermented products such as yogurt, cheese and other fermented milk products (Salminen, 1996). Their unique presence in intestinal epithelium and human gastrointestinal tract, and their traditional use in fermented foods and dairy products without remarkable problems prove their safety (Pangallo et al., 2008). The aim of this study was to isolate and identify probiotic lactic acid bacteria from curd and evaluate *in vitro*, its growth inhibition activities against pathogenic bacteria.

## MATERIALS AND METHODS

### Isolation of lactic acid bacteria from curd

Lactic acid bacteria were isolated from curd obtained from different sources of dairy products. Samples were collected using sterile bottles and stored in an icebox until delivery to the laboratory for

analysis. The curd samples were suspended appropriately and diluted in sterile saline and spread plated into selective medium: De Mann, Rogosa Sharpe (MRS) (Dave and Shah, 1996) and incubated at 37°C for 24 h anaerobically for isolation. The selected colonies were purified by streak plate technique. The purified bacterial strains were stored at -80°C in sterile reconstituted skim milk 12.5% (w/v) supplemented with 15% glycerol.

### Physiological and biochemical characterization of lactic acid bacteria

Growth of lactic acid bacteria (LAB) isolates was examined in MRS broth at temperature 15, 37 and 45°C by incubating for 24 h anaerobically (Briggs, 1953). CO<sub>2</sub> and lactic acid production from glucose was tested in citrate lacking MRS broths media containing inverted Durham tubes. Salt tolerance was tested in MRS broth by incorporating at 2, 4 and 6.5% (w/v) sodium chloride, incubated at 37°C was carried out according to method described by Briggs (1953). Catalase test was performed by adding 3% of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in a test tube containing an overnight culture of lactic acid bacteria. Ammonia production in MRS broth containing 0.3% arginine and 0.2% sodium citrate instead of ammonia citrate and the production of ammonia was detected using Nessler's reagent (Briggs, 1953). Sugar fermentation test was performed using eight different sugars (mellibiose, cellobiose, lactose, sucrose, raffinose, gluconate, salicine and arabinose) as reported by Cullimore (2008). Gram positive, catalase negative and bacilli colonies were taken as lactic acid bacteria and stored in glycerol culture and kept at -80°C for further investigation.

### Survival of lactic acid bacteria isolates under conditions simulating human GI tract

Major selection criteria (antibiotic resistance, resistance to low pH, tolerance against bile salt, bile salt hydrolysis and antimicrobial activity) were used for the determination of probiotic properties of lactic acid bacteria isolates (Bassyouni et al., 2012).

### Antibiotic resistance

Lactic acid bacteria strains were assessed their antibiotic resistance by disc diffusion method using antibiotics discs. One milliliter of actively growing cultures was mixed with 10 ml of MRS agar and poured into a petriplates. After solidification, the antibiotic disks were placed on the solidified agar surface, and the plates were left over for 30 min at 4°C for diffusion of antibiotics and then anaerobically incubated at 37°C for 48 h. Resistance was defined according to the disc diffusion method by using antibiotic discs of streptomycin, gentamycin, chloramphenicol, ciprofloxacin, norfloxacin and tetracycline (Hi Media Laboratories Pvt. Ltd. Mumbai, India.) (Felten et al., 1999). The zone of inhibition was measured by calipers in millimeters (Lleó et al., 1998; Mir-hoseini, 2004).

### Resistance to low pH

Lactic acid bacteria isolates obtained from overnight culture were harvested by centrifugation for 10 min at 5000 rpm and 4°C, washed twice with PBS buffer (pH 7.2), and adjusted to pH 2.5. Resistance were assessed in triplicates in terms of viable colony counts and enumerated on MRS agar after incubation at 37°C for 0, 1, 2 and 3 h, indicating the time spent by food in the stomach as described by Maragkoudakisa et al. (2006) and Zoumpopoulou et al. (2008). Also, growth was monitored at OD<sub>620</sub> (Bassyouni et al., 2012).

### Resistance to bile salt

The tolerance against bile was carried out based on the intestinal bile concentration, 0.3% (w/v) and the staying time of food in small intestine is 4 h (Kumar and Murugalatha, 2012). MRS medium containing 0.3% (w/v) bile concentration was inoculated with overnight culture of lactic acid bacteria. Viable colonies were counted for every hour of incubation time on MRS agar and also growth was monitored at OD<sub>620</sub> (Bassyouni et al., 2012).

### Bile salt hydrolysis

Bacterial cultures were grown overnight in MRS broth at 37°C with 5% CO<sub>2</sub> and streaked in triplicates on MRS agar containing 0.5% (w/v) taurodeoxycholic acid. The hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of anaerobic incubation at 37°C.

### Antimicrobial activity against bacterial pathogens

The lactic acid bacteria isolates were tested for antagonistic activity against five bacterial pathogens (*Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E. coli* sp.) (Mir-hoseini, 2004) was obtained from Haramaya University, Microbiology Laboratory. The lactic acid bacteria strains were inoculated in MRS broth for 24 h at 37°C and centrifuging the culture at 5000 rpm for 10 min at 4°C, and the bacterial cells were removed. The pH values of supernatants were neutralized to 6.5 by the addition of 0.1 N NaOH, and filtered through a sterilized cellulose acetate filter and stored at 4°C. Supernatants were directly used for antagonistic test. Pellet was diluted with sterile distilled water and used for antimicrobial activity test.

### Antimicrobial assay

The antimicrobial activity of the lactic acid bacteria isolates was determined by using the disc diffusion method as described by Sandra et al. (2012). These assays were performed in triplicate. The plates were poured with 20 ml Nutrient Agar. The pathogenic strains (*Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E. coli* sp.) were spread on the surface of nutrient agar. The agar plates inoculated with test organism were incubated for 1 h before placing the extract impregnated paper discs on the plates. Five sterile papers blank disks of 6 mm diameter were placed on the surface of agar plate which was inoculated by indicator strains of 5, 10 and 15 µl of the filtered supernatant and pellets of *Lactobacilli* were applied for screening of antimicrobial activity. The plates were kept at 4°C for 30 min to permit diffusion on the assay material, and incubated at 37°C for 24 h. Discs dipped in sterile water served as control and the antibiotics Chloramphenicol, Penicillin G and Streptomycin were used as positive control. Zones of inhibition were measured by calipers in millimeters (Lleó et al., 1998; Mir-hoseini, 2004).

### Haemolytic activity

Fresh overnight bacterial cultures was streaked in triplicates on Columbia agar plates, containing human blood and incubated at 30°C for 48 h. Haemolytic activities of the bacterial culture were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green zones around colonies) or γ-haemolysis (no clear zones around colonies) on human blood agar plates (Hargrove and Alford, 1978).

## RESULTS AND DISCUSSION

### Isolation of lactic acid bacteria (LAB)

Nine *Lactobacillus* strains were isolated from curd and based on their physiological and biochemical characteristics isolates were designated as: A, B, C, D, E, F, G, H and I.

### Physiological and biochemical identification

According to biochemical characterization, all tested isolates were grown at 37, 45°C where as isolate B, D, F, and I were grown at 15°C. All tested isolates did not produce ammonia from arginine and did not produce gas from glucose. All isolates tolerated 2, 4 and 6.5% of NaCl concentration and was catalase negative. The recognition of lactobacillus strains was made mainly on the results of carbohydrate fermentation tests. According to carbohydrate fermentation, isolate A ferment cellobiose, lactose, sucrose and salicine. Isolate B ferment sugar lactose, sucrose, gluconate and salicine. Isolate C ferment only sucrose. Isolate D ferments sugars mellibiose, gluconate and arabinose. Isolate E ferment carbohydrates mellibiose, sucrose, raffinose and gluconate. Isolate F ferment cellobiose, lactose, sucrose and salicine. Isolate G ferment carbohydrates cellobiose, lactose, sucrose, salicine. Isolate H ferment lactose, sucrose, salicine, and isolate I ferment mellibiose, cellobiose, gluconate, salicine and arabinose carbohydrates. Based on the biochemical characterization, isolate A is *Lactobacillus leichmannii*, isolate B: *Lactobacillus casei*, isolate C: *Lactobacillus delbrueckii*, isolate D: *Lactobacillus brevis*, isolate E: *Lactobacillus fermentum*, isolate F: *Lactobacillus coagulans*, isolate G: *Lactobacillus acidophilus*, isolate H: *Lactobacillus lactis* and isolate I: *Lactobacillus rhamnosus*. This in agreement with Karimi et al. (2012) and Liu et al. (2012) who isolated *L. acidophilus* from cheese and found that *L. lactis* and *L. casei* were considered as the predominated species in fermented dairy products (Tables 1 and 2).

### Survival of lactic acid bacteria isolates under conditions simulating human GI tract

In order to use lactic acid bacteria as a probiotic, the bacteria must be able to survive the acidic conditions in the stomach and resist bile acids concentration (Holzapfel et al., 1998; Klaenhammer and Kullen, 1999) and possess antagonistic effect against pathogenic bacteria (Mir-hoseini, 2004).

### Resistance to low pH

Being resistant to low pH is one of the major selection criteria for probiotic strains (Çakır, 2003). To reach

**Table 1.** Morphological and cultural characteristics of *Lactobacillus* isolates.

Laboratory isolate	Cell morphology	Spore forming	Capsule	Motility	Growth at			CO <sub>2</sub> production	Ammonia production	Catalase test
					15°C	37°C	45°C			
A	SR	-	-	-	-	+	+	-	-	-
B	SR	-	-	-	+	+	+	-	-	-
C	SR	-	-	-	-	+	+	-	-	-
D	SR	-	-	-	+	+	+	-	-	-
E	SR	-	-	-	-	+	+	-	-	-
F	SR	-	-	-	+	+	+	-	-	-
G	SR	-	-	-	-	+	+	-	-	-
H	SR	-	-	-	-	+	+	-	-	-
I	SR	-	-	-	+	-	-	-	-	-

<sup>SR</sup>Straight rods, <sup>v</sup>variable, <sup>-</sup>negative, and <sup>+</sup>positive.

**Table 2.** Carbohydrate fermentation of lactic acid bacteria.

Laboratory isolate	Melibiose	Cellobiose	Lactose	Sucrose	Raffinose	Gluconate	Salicine	Arabinose	Strain
A	-	+	+	+	-	-	+	-	<i>L. leichmannii</i>
B	-	-	+	+	-	+	+	-	<i>L. casei</i>
C	-	-	-	+	-	-	-	-	<i>L. delbrueckii</i>
D	+	-	V	v	V	+	-	+	<i>L. brevis</i>
E	+	-	+	+	+	+	-	V	<i>L. fermentum</i>
F	-	+	+	+	-	-	-	+	<i>L. coagulans</i>
G	V	+	+	+	V	-	+	-	<i>L. acidophilus</i>
H	-	-	+	+	-	-	+	-	<i>L. lactis</i>
I	+	+	V	-	-	+	+	+	<i>L. rhamnosus</i>

\* Positive, <sup>-</sup>negative, and <sup>v</sup>variable.

the small intestine they have to pass through stressful conditions of the stomach (Çakir et al, 2003). Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad et al., 1998), for selection of the strains resistant to low pH, PBS pH was adjusted to 3.0. The time taken during the digestion in the stomach is 3 h. So, all the isolates were detected whether they were resistant to pH 3.0 during 3 h or not. All the tested isolates were able to survive at pH 3.0 during three hours of incubation. *L. casei* and *L. delbrueckii* were more resistant to low pH than other strains. This correlated with the previous report by Argyri et al. (2013) were nine strains of *Lactobacillus* showed very high resistance to low pH (*L. plantarum*, *L. pentosus*, *L. paracasei* subsp. *paracasei*) with final populations exceeding 8 log cfu/ml, whereas 12 strains showed high resistance to low pH (*L. plantarum*, *L. pentosus*) which along with the reference strains *L. casei* and *L. rhamnosus* GG showed final populations within 6-8 log cfu/ml and according to Bassyouni et al. (2012), eight of eleven tested lactobacillus isolates were resistance to pH 3.0 during three hours. All isolates that survive in pH 3.0

were taken to the next step for further investigation (Table 3).

**Table 3.** Survival in pH 3.0 - OD620 value.

Laboratory isolate	OD at 620nm at different time interval (hour)			
	0	1	2	3
B	1.1	1.1	1	1.2
C	0.99	1	0.98	1
D	0.71	0.7	0.7	0.7
H	0.56	0.58	0.59	0.58
I	0.4	0.38	0.35	0.3

### Resistance to bile salt and bile salt hydrolysis

The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time is suggested to be 4 h (Prasad



et al., 1998). Strains were detected in 0.3% during 4 h of stay. Results showed that all isolates after four of exposure retaining their viability with negligible reduction in viable counts to 0.3% (w/v) bile concentration and among all of tested isolates, *L. casei* and *L. delbrueckii* were more tolerant than others. This in agreement with Jensen et al. (2012) report that *Lactobacillus* species tolerate gastric juice well with no reduction in viability. Similarly observation by Abriouel et al. (2012), showed all lactic acid bacteria isolated from fermented olive were able to grow and survive at 0.3% (w/v) bile salt. The survival at bile salt condition is one of the main criteria for *in vitro* selection of potentially probiotic bacteria and critical points for the microbes. Regarding the bile salt hydrolysis, *L. casei* and *L. delbrueckii* show partial bile salt hydrolysis activity and colony morphology was recorded as differentiated in comparison with the control MRS agar plates (Table 4).

**Table 4.** Tolerance against 0.3% bile – OD620 values.

Laboratory isolate	OD at 620nm at different time interval (hour)				
	0	1	2	3	4
B	0.055	0.042	0.049	0.049	0.07
C	0.038	0.036	0.037	0.04	0.042
D	0.031	0.03	0.029	0.031	0.032
H	0.02	0.023	0.03	0.028	0.031
I	0.03	0.028	0.025	0.02	0.018

### Haemolytic activity

Non-haemolytic activity and antibiotic resistance strains are considered as a safety prerequisite for the selection of a probiotic strain. Results showed that all examined strains did not exhibited  $\beta$ -haemolytic activity when grown in Columbia human blood agar. Most of the strains (7 strains) were  $\gamma$ -haemolytic (non-haemolysis), while two strains exhibited  $\alpha$ -haemolysis. These were *L. coagulans*, *L. rhamnosus*. Isolate *L. casei*, *L. delbrueckii* and *L. lactis* showed  $\gamma$ -haemolysis (Maragkoudakisa et al., 2006).

### Antimicrobial activity

The cell free supernatants and cell masses of different lactobacillus strains were tested for antimicrobial activity against five pathogenic bacterial strains (*Staphylococcus* sp., *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *E. coli* sp.) by using disc diffusion method. The production of antimicrobial compounds such as organic acids, short chain fatty acids and bacteriocins is one of the functional properties used to characterize probiotics (Fuller, 1989). The capacity to produce different antimicrobial compounds may be one of the critical characteristics for effective competitive exclusion of pathogen survival in the intestine and expression of a probiotic effect for the host

(Salminen et al., 1998). In this study, the antimicrobial activities of supernatants and cell masses were conducted at three different concentrations of 5, 10 and 15  $\mu$ l/discs were inoculated and compared with those of positive control such as tetracycline, penicillin G and streptomycin and distilled water as negative control. The data obtained from the disc diffusion method indicated that the extract displayed a variable degree of antimicrobial activity on different tested strains. The inhibitory effect was increased with the increase of the extract concentration from 5 to 15  $\mu$ l. The diameters of inhibition zones were measured and compared. Result showed that *L. casei* and *L. delbrueckii* showed maximum inhibition zone against bacterial pathogens among all the *Lactobacillus* isolates. This in agreement with Bassyouni et al. (2012) that *Lactobacillus* strains tested against *Salmonella thyphimurium*, *E. coli*, and *Staphylococcus* spp. and all of tested isolates have antibacterial effect against the pathogenic bacteria (Tables 5 and 6).

### Resistance to antibiotics

Resistance was defined according to the standard disc diffusion method by using antibiotic discs of Streptomycin, Gentamycin, Chloramphenicol, Ciprofloxacin, Norfloxacin and Tetracycline (Hi Media Laboratories Pvt. Ltd. Mumbai, India.). The zone of inhibition was measured by calipers in millimeters (Lleó et al., 1998; Mir-hoseini, 2004). Results showed that *L. casei*, *L. delbrueckii* and *L. brevis* were resistant to all antibiotics and *L. fermentum*, *L. lactis* and *L. rhamnosus* were sensitive to all antibiotics except Streptomycin. *L. leichmannii*, *L. acidophilus* and *L. coagulans* were sensitive to all antibiotics. *L. casei*, *L. delbrueckii*, *L. lactis*, *L. rhamnosus* and *L. brevis* were taken to the next step for further investigation (Table 7).

### Conclusions

The *Lactobacillus* cultures were isolated and identified according to their physiological and biochemical characteristics. The used tests were: lactic acid and CO<sub>2</sub> production test, ammonia production test, catalase test, growth at 15, 37 and 45°C, salt tolerance test and sugar fermentation test. Results showed that the strains *L. leichmannii*, *L. casei*, *L. delbrueckii*, *L. brevis*, *L. fermentum*, *L. coagulans*, *L. acidophilus*, *L. lactis* and *L. rhamnosus* were isolated from curd. *Lactobacillus* strains survival was also assessed under conditions simulating human GI tract which were performed followed by antibiotic resistance, resistance to bile salt, bile salt hydrolysis and resistance to low pH. Results showed that all isolates were able to grow at low pH 3.0, at 0.3% bile concentration. *L. casei*, *L. delbrueckii* and *L. brevis* were found to be resistant to five antibiotics, that is, Tetracycline, Streptomycin, Gentamycin, Ciprofloxacin and Norfloxacin. According to haemolytic activity all

**Table 5.** Antimicrobial activity of lactic acid bacteria cell mass.

Laboratory isolate	Volume of cell mass ( $\mu$ l)	Diameter of inhibition zone (mm)				
		<i>E. coli</i>	<i>Pseudomonas</i>	<i>Staphylococcus</i>	<i>Klebsiella</i>	<i>Bacillus</i>
B	5	-	11	10	9	8
	10	10	13	12	12	9
	15	10	20	14	15	12
C	5	8	-	8	-	-
	10	10	7	11	-	-
	15	11	10	14	-	10
D	5	-	-	-	8	-
	10	10	-	-	10	-
	15	10	-	-	10	12
H	5	-	-	-	-	-
	10	8	-	-	-	-
	15	11	-	-	-	-
I	5	8	-	-	-	-
	10	10	-	-	-	-
	15	13	10	9	-	-

**Table 6.** Antimicrobial activity of lactic acid bacterial supernatant.

Laboratory isolate	Different volume of supernatant mass ( $\mu$ l)	Diameter of inhibition zone(mm)				
		<i>E. coli</i>	<i>Pseudomonas</i>	<i>Staphylococcus</i>	<i>Klebsiella</i>	<i>Bacillus</i>
B	5	-	-	9	8	-
	10	10	8	11	9	10
	15	12	10	12	10	10
C	5	-	10	-	7	8
	10	8	12	8	10	10
	15	9	15	9	14	13
D	5	-	-	7	-	-
	10	10	-	8	-	-
	15	11	-	10	-	-
H	5	-	7	7	-	-
	10	8	10	9	10	8
	15	11	12	1	15	9
I	5	-	-	-	-	-
	10	-	-	-	-	-
	15	-	-	-	-	-

examined strains did not exhibited  $\beta$ -haemolytic activity when grown in Columbia human blood agar. Most of the strains (7 strains) were  $\gamma$ -haemolytic (non-haemolysis), while two strains exhibited  $\alpha$ -haemolysis. These were *L. coagulans*, *L. rhamnosus*. Isolate *L. casei*, *L. delbrueckii* and *L. lactis* showed  $\gamma$ -haemolysis. Regarding the bile salt hydrolysis, *L. casei* and *L. Delbrueckii* show partial bile salt hydrolysis activity and colony morphology was recorded as differentiated in comparison with the control MRS agar plates. Finally, antimicrobial activities of

*Lactobacillus* isolates were tested against five pathogenic bacteria (*Staphylococcus* spp., *Bacillus* spp., *Klebsiella* spp., *Pseudomonas* spp., and *E. coli* spp.) at pH 6.5 by disc diffusion method. All the tested isolates showed *in vitro* inhibitory zone against pathogenic bacteria at pH 6.5. Among the *Lactobacillus* strains, *L. casei*, and *L. delbrueckii* showed maximum inhibition zone. In conclusion, the present study showed that from all the isolated *Lactobacillus* strains, *L. casei* and *L. delbrueckii* can be used as potential probiotic lactic acid bacteria.

**Table 7.** Drug resistance of *Lactobacillus* strains.

Laboratory isolate	Streptomycin	Tetracycline	Gentamycin	Norfloxacin	Ciprofloxacin
A	12	25	20	26	25
B	R	R	R	R	R
C	R	R	R	R	R
D	R	R	R	R	R
E	R	25	R	10	10
F	14	40	25	30	35
G	10	40	20	35	35
H	R	12	15	17	R
I	R	25	16	10	15

<sup>R</sup>Resistant.

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Full Length Research Paper

## Bacterial diversity in the rumen of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet

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This work was done to study the bacterial diversity of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet. Genomic DNA was extracted from the rumen liquor of mithun, 16S rDNA sequences were amplified, cloned and randomly selected for sequencing. The nearest neighbors were retrieved from the NCBI through a BLAST search and a phylogenetic tree was constructed. In our findings, 12% of clones showed similarity with known bacterial species (*Prevotella ruminicola*, *Butyrivibrio fibrisolvens*, *Pseudobutyrvibrio ruminis*, *Succinivibrio dextrinosolvens* and *Ruminococcus flavefaciens*) and 6% of clones showed similarity with known bacterial genus (*Butyrivibrio* species, *Streptococcus* species) of 97-100% similarities. Twenty-two percent of clones showed similarity with known bacteria (*P. ruminicola*, *Prevotella* species, *Sporanaerobacter acetigenes*, Clostridiales bacterium, Bacteroidetes bacterium) of 90-97% similarities. Sequences of all the clones were also classified by using taxonomic classifier software available at Ribosomal Database Project and classification showed that, all the clones were under four phyla, namely Bacteroidetes (54%), Firmicutes (36%), Proteobacteria (4%) and Tenericutes (2%). The experiment showed that, bacterial population in the rumen of mithun fed on mixed tree leaves and rice straw based diet harbor diversified species of bacteria responsible for lignocellulosic feedstuffs.

**Key words:** Tree leaves, bacteria, clones, sequences.

### INTRODUCTION

The North-East India, being at the confluence of three major bio-geographical realms of the world, is extremely rich in floral and faunal biodiversity with several endemic species. Among these, mithun (*Bos frontalis*) is considered as the most important bovine species and the people not only use them as pride object of social

sacrifice but as life currency in their local transactions (Annual Report, 2012). This unique bovine species is believed to be domesticated more than 8000 years ago and is mainly available in the four north-eastern hilly states of Arunachal Pradesh, Nagaland, Mizoram and Manipur. It plays an important role in economic, social

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**Table 1.** Chemical composition of feed and fodder (percentage on DM basis) fed to mithun during the experiment.

Item	Concentrate mixture	<i>Ficus hirta</i>	<i>Debrogesia longifolia</i>	Litsea sps	<i>Legroestromea spaciola</i>	Mixed tree leaves calculated	Paddy straw
DM	89.25	23.6	28.0	35.14	36.81	30.88	87.30
OM	91.11	93.8	89.8	93.70	94.99	93.07	85.92
CP	17.71	15.6	14.0	14.60	14.32	14.63	4.36
EE	8.35	1.8	1.8	2.80	2.14	2.14	1.08
CF	9.70	18.3	12.8	16.30	26.53	18.48	33.79
NFE	55.35	58.1	61.2	60.0	52.0	57.82	46.69
TA	8.89	6.2	10.2	6.3	5.01	6.93	14.08

and cultural life of the tribal people of this region. It is primarily reared as a meat animal and is highly preferred among the tribal people of the region. This animal is reared exclusively under free grazing condition. Mithun is an extremely efficient grazer on steep hilly slopes as compared to other animals. It basically thrives on the jungle forages, tree fodders, shrubs, herbs and other natural vegetation. It prefers to browse and move around the forest in search of selective forages. Farmers do not provide any additional supplement except for occasional common salt feeding, especially at the time of restraining for some purposes (Moyong, 2012). The performance of this species of animal was also found to be satisfactory in confinement when reared on tree leaves based ration (Das et al., 2010).

Rumen microbes have been extensively studied in ruminants like cattle and buffaloes both qualitatively (Koike et al., 2003; Sylvester et al., 2004) and quantitatively (Shin et al., 2004) using DNA-based technologies (16s RNA/18s RNA gene). These techniques have been further used to construct a library of 16S rDNA clones of rumen microbes and to demonstrate considerable diversity of rumen bacteria. The microbes present in the rumen ecosystem of mithun convert the tree leaves and shrubs rich in lignocellulosic materials into volatile fatty acids and microbial protein for the animals (Das et al., 2010). The diversity study of rumen bacteria in mithun will provide sufficient information for rumen manipulation in future for improving growth and production. In the present experiment, the rumen bacterial diversity of mithun were studied, fed on mixed tree leaves and rice straw based diet by amplification, cloning and sequencing of 16S rRNA gene of bacteria, followed by sequence comparison and phylogenetic analysis.

## MATERIALS AND METHODS

### Experimental animals

The experiment was carried out on five adult mithun about 3 years of age at Research Farm of National Research Centre on Mithun,

Jharnapani, Medziphema, Nagaland, India. The diet consisted of mixed tree leaves, paddy straw and concentrates mixture (Table 1). Approximately 50% of dry matter (DM) requirement was met through concentrate mixture and rest through mixed tree leaves and paddy straw (2:1 ratio on fresh basis) according to the standard developed in the institute. The tree leaves consisted of temechiedie (*Ficus hirta*), Pedu (*Debrogesia longifolia*), thenha (Litsea sps) and thumero (*Legroestromea spaciola*). Leaves of these tree foliage were cut, carried daily, mixed in equal proportions and fed to the experimental animals. Fresh drinking water was offered *ad lib* two times a day. All the animals were maintained on uniform feeding regime for a period of one year.

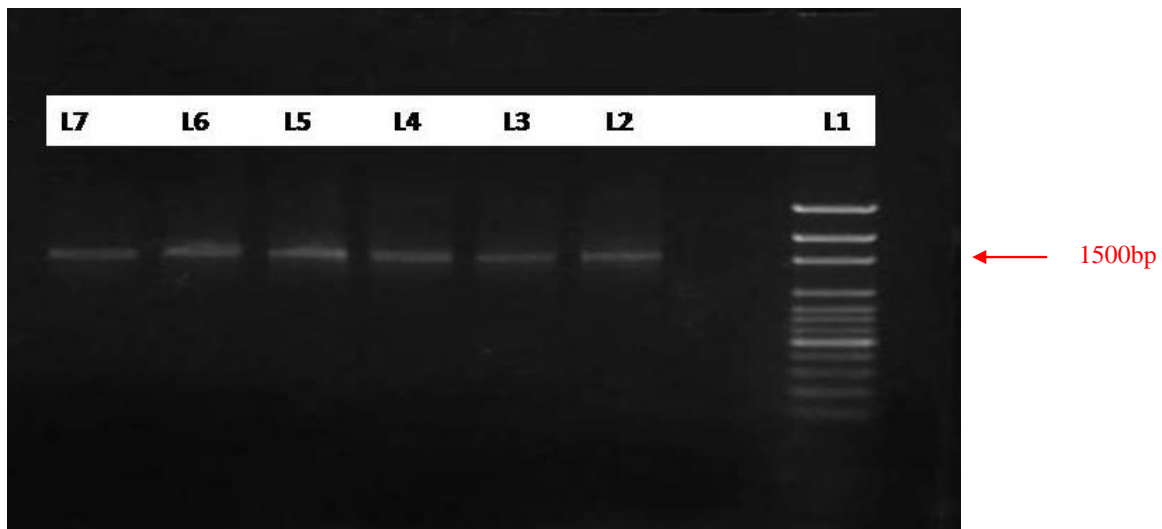
### Rumen sample collection

Approximately 50 ml of rumen fluid from each animal was collected via a stomach tube located in the middle part of the rumen and connected to a vacuum pump at 3 h post feeding. Samples were pooled and filtered through four layers of muslin cloth to remove particulate matter. Strained samples were used in the laboratory for the total bacterial DNA extraction.

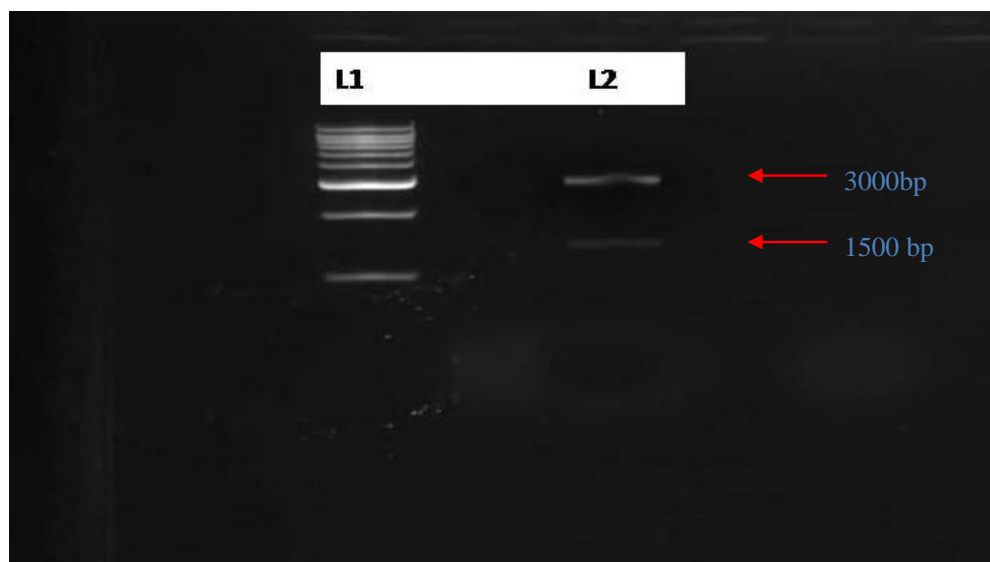
### DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from the mixed rumen liquor by using the standard kit manufactured by Bangalore Genei India Pvt. Ltd., Peenya, Bangalore, India. The DNA were checked by agarose gel electrophoresis and then polymerase chain reaction (PCR) amplification of bacterial 16S rDNA was performed using the universal primer of bacteria F27(5'-AGATTGATCMTGGCTAGGGA-3') and R1492 (5'-TACGGYTACCTTGTACGACTT-3') as reported by Weisburg et al. (1991). The PCR reaction was set up in 25 µl volumes containing 1 µl template, 2.5 µl 10x buffer, 1.5 µl 25 mM MgCl<sub>2</sub>, 1 µl of each primer, 0.5 µl of 25 mM dNTP mix, 0.5 µl Taq DNA polymerase and distilled water. The amplification conditions are standardized for universal primer. The amplification conditions were as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 2 min with the last cycle followed by a 10 min extension step at 72°C. The PCR product was visualized on an agarose gel (Figure 1), the bands were excised and DNA was purified from the gel slices using the standard kit manufactured by Bangalore Genei India Pvt. Ltd., Peenya, Bangalore, India. The purified PCR product was stored at -20°C for further processing.

The purified PCR products were ligated into T vector using ligation kit manufactured by Bangalore Genei (India) Pvt. Ltd according to the instruction and then transferred into *E. coli*, DH5.



**Figure 1.** Agarose gel electrophoresis of PCR product after amplification with universal primer of bacteria (L1: 1 kb DNA marker, L2-L7: test samples).



**Figure 2.** Agarose gel electrophoresis of PCR product showing restriction enzyme digestion of Plasmid DNA (L1: DNA marker, L2: test sample).

Recombinant cells were allowed to grow on LB medium containing ampicillin, IPTG and X gal for overnight and then the white colonies were picked up. The extraction of recombinant plasmid was carried out by using plasmid extraction kit manufactured by Himedia Laboratories, Mumbai (HiPura Plasmid DNA Minispin Purification Spin Kit). The restriction enzyme digestion of plasmid DNA was carried out to confirm the identity of PCR products (Figure 2). Sequencing of clones was performed with an ABI prism genetic Analyser by Bioserve, Hyderabad. The nearest neighbors were retrieved from the NCBI (<http://www.ncbi.nih.gov/BLAST>) through a BLAST search.

#### Sequence analysis and construction of phylogenetic tree

Sequences from the current study were analyzed by the CHECK\_CHIMERA program (Maidak et al., 2001) to remove any chimeric rDNA clone. Sequence alignment was achieved using multiple sequence alignment software CLUSTAL W Version 1.81 (Thompson et al., 1994). The criterion used to define a clone sequence as being that for a particular species of rumen bacteria was that the similarity of the sequence should be 97% or greater with that of the known species (Stackebrandt and Goebel, 1994). The sequences of the isolates were compared with those available

in the database. The obtained sequences were aligned using clustal V method of megAlign software (DNASTAR) and then phylogenetic tree was plotted.

## RESULTS AND DISCUSSION

### Chemical composition of ration

The chemical composition feed and fodder during the experimental period is given in Table 1. The crude protein content of concentrate mixture, mixed tree leaves and paddy straw was estimated to be 17.71, 12.01 and 4.36%, respectively. The crude protein content of tree leaves/shrubs reported in this experiment is comparatively higher than the green fodder of other parts of India. The mithun is reared on tree leaves available in the forest of north-east region of India whereas cattle and buffaloes are normally fed on cultivated green fodder. Hence, there is provision of excellent vegetation for the mithun in this area as compared to cattle and buffaloes in other parts of India.

### Similarity of sequences

A total of 100 clones were isolated from the mixed rumen liquor of mithun (*Bos frontalis*) and 72 of the clones were randomly selected for sequencing. All sequences were checked for vector sequence contamination and then submitted to GenBank in NCBI. The result of similarity values of clones (16S rDNA sequences) is presented in Table 2. In our findings, 9 clones (12% of clones) showed similarity with known bacterial species (*Prevotella ruminicola* 4, *Butyrivibrio fibrisolvens* 2, *PseudoButyrivibrio ruminis* 1, *Succinivibrio dextrinosolvens* 1 and *Ruminococcus flavefaciens* 1) and 4 clones (6% of clones) showed similarity with known bacterial genus (*Butyrivibrio* species 3, *Streptococcus* sp 1) of 97-100% similarities. Identification using sequence similarity demands sequences having similarity more than 97%. Many sequences showed similarity value of less than 97%, thus confirming their difference with known sequences at species level. Sixteen of the clones (22%) showed similarity with known bacteria of 90-97% similarities (*Prevotella ruminicola* 7, *Prevotella* species 4, *Sporanaerobacter acetignes* 1, Clostridiales bacterium 2, Bacteroidetes bacterium 2). Forty three (60%) of clones in this study were uncultured rumen bacterium.

Sequences of all the clones were also classified by using taxonomic classifier software available at Ribosomal Database Project. The sequences were submitted to the software and classification showed that, all the clones were under four phyla, namely Bacteroidetes (54%, 39 clones), Firmicutes (36%, 26 clones), Proteobacteria (4%, 3 clones) and Tenericutes

(2%, 1 clones). Four percent (three clones) were unidentified bacteria in this study. The majority of bacteria were from the genus *Prevotella* (Phylum- Bacteroidetes) as they have a very significant role in the digestion of feed stuffs of rumen. Under the phylum Firmicutes, the bacteria of family Succinivibrio, Ruminococcaceae, Lachnospiraceae, Streptococcaceae and Enterococcaceae were identified. *Succinivibrio* and *Vampirovibrio* are the two types of bacteria (genus) identified under the phylum Proteobacteria. This work was similar to the findings of Patel (2011) who revealed through the Ribosomal Database Project (RDP) classification that, the clones of rumen in goat were mainly distributed into two phyla, namely Bacteroidetes (35.0%) and Firmicutes (33.0%). In contrast to these findings, Tajima et al. (1999) reported that 52.4% of clones identified in the rumen of Holstein cow fed a diet of hay belonged to the firmicutes, and 38.1% to the Cytophaga-Flexibacter-Bacteroides (CFB) phylum. Other studies (Edwards et al., 2004; Deng et al., 2007) reported almost the same experimental findings of Tajima et al. (1999) who worked on the microbes of ruminant animals.

### Phylogenetic analysis

The similarity for most of the sequences with those of known rumen bacteria was too low for accurate identification of the sequence. Therefore, a phylogenetic tree was constructed using MEGA version 5 software to investigate the taxonomic placement. The results of this phylogenetic analysis are shown in Figure 3. The phylogenetic tree was mainly divided into two clusters, cluster I and II. Cluster I is again divided into five sub-groups. In sub-group I, 13 clones are grouped separately out of which four clones are *Butyrivibrio* species (NRCMK61, NRCMK50, NRCMK46 and NRCMK49), one clone (NRCMK60) is *Pseudobutyrvibrio* species and the remainder are uncultured rumen bacteria. Sub-group II consisted of 12 clones out of which, 5 clones (NRCMB1, NRCMK31, NRCMK15, NRCMK17 and NRCMK23) are of *Prevotella* species, two clones of Bacteroidetes bacterium (NRCMK29 and NRCMK65) and the remaining 5 clones are of uncultured bacteria. In sub-group III, 14 clones are grouped together and most of the clones (10) in this group are of uncultured rumen bacteria, one clone is *Ruminococcus flavefaciens* (NRCMK70), one clone is *Streptococcus* sp. (NRCMK47) and two Clostridiales bacterium (NRCMK10 and NRCMK13). In sub-group IV, 10 clones are grouped together and all the clones in this sub-group are uncultured rumen bacteria. One clone (NRCMK22) is separated from all the sub-groups of cluster I which may be termed as unidentified bacteria. Similarly, In Cluster II, 22 clones are grouped together out of which two clones (NRCMK64 and NRCMK57) are separated from others. Remaining 20 clones are grouped



**Table 2.** Similarity values of clones (16S rDNA sequences) retrieved from the rumen fluid of mithun.

Clone no.	Accession no (query sequence)	Nearest valid relatives	Accession no (subject sequence)	Identity
NRCM K10	JX855075	Clostridiales bacterium	AB702858	95
NRCM K18	JX855076	Uncultured rumen bacterium clone	GQ327497	93
NRCM K7	JX855077	Uncultured rumen bacterium clone	HM008741	94
NRCM K11	JX855078	Uncultured rumen bacterium clone	EF436438	94
NRCM K28	JX855079	Uncultured rumen bacterium clone	GU302718	95
NRCM K36	JX855080	Prevotella sp. clone	DQ308605	96
NRCM K34	JX855081	Uncultured rumen bacterium clone	EU842835	98
NRCM K17	JX855082	Prevotella ruminicola	AB501167	95
NRCM K21	JX855083	Uncultured rumen bacterium clone	AB270090	99
NRCM K4	JX855084	Uncultured rumen bacterium clone	EF445288	96
NRCM K33	JX855085	Prevotella ruminicola	AB501171	97
NRCM K31	JX855086	Prevotella ruminicola	AB501153	95
NRCM K15	JX855087	Prevotella ruminicola	AB501173	94
NRCM K13	JX855088	Clostridiales bacterium	GQ358492	91
NRCM K24	JX855089	Prevotella ruminicola	AB501172	96
NRCM K1	JX855090	Uncultured rumen bacterium clone	DQ673507	96
NRCM K25	JX855091	Uncultured rumen bacterium clone	GQ327548	97
NRCM K19	JX855092	Uncultured rumen bacterium clone	GQ327497	90
NRCM K40	JX855093	Uncultured rumen bacterium clone	EF445285	97
NRCM K20	JX855094	Uncultured rumen bacterium clone	AB034128	93
NRCM K30	JX855095	Prevotella ruminicola	CP002006	99
NRCM K9	X855096	Uncultured rumen bacterium clone	EF686573	95
NRCM K23	JX855097	Prevotella ruminicola	AB501173	96
NRCM K39	JX855098	Uncultured rumen bacterium clone	EU461449	98
NRCM K5	JX855099	Uncultured rumen bacterium clone	EF445288	96
NRCM K14	JX855100	Uncultured rumen bacterium clone	EU719218	90
NRCM K37	JX855101	Prevotella ruminicola clone	AB501168	93
NRCM K12	JX855102	Uncultured rumen bacterium gene	AB185591	95
NRCM K3	JX855103	Uncultured rumen bacterium clone.	EU842595	99
NRCM K26	JX855104	Uncultured rumen bacterium clone	EU259500	97
NRCM K8	X855105	Uncultured rumen bacterium clone	EU778030	96
NRCM K16	JX855106	Uncultured rumen bacterium clone	GU302850	96
NRCMK 29	JX855107	Bacteroidetes bacterium clone	HM104872	93
NRCM K6	JX855108	Uncultured rumen bacterium clone	FJ684957	90
NRCM K2	JX855109	Uncultured rumen bacterium clone	EF436384	98
NRCM K35	JX855110	Prevotella ruminicola	AB501163	98
NRCM K22	JX855111	Uncultured rumen bacterium clone	AB270037	97
NRCM K38	JX855112	Uncultured rumen bacterium clone	GQ327819	98
NRCM K32	JX855113	Prevotella ruminicola	AY699286	98
NRCM K27	JX855114	Uncultured rumen bacterium clone	AY838556	97
NRCM K45	KC020714	Uncultured rumen bacterium clone	EU844417	96
NRCM K53	KC020715	Uncultured rumen bacterium clone	JF633438	98
NRCM K51	KC020716	Uncultured rumen bacterium clone	EU459560	94
NRCM K46	KC020717	Butyrivibrio fibrisolvens	EU684229	99
NRCM K52	KC020718	Uncultured rumen bacterium clone	HQ399710	93
NRCM K44	KC020719	Uncultured rumen bacterium clone	AY854313	97
NRCM K58	KC020720	Uncultur Uncultured rumen bacterium clone	DQ673473	95
NRCM K55	KC020721	Uncultured rumen bacterium clone	JF635508	98

Table 2. Contd.

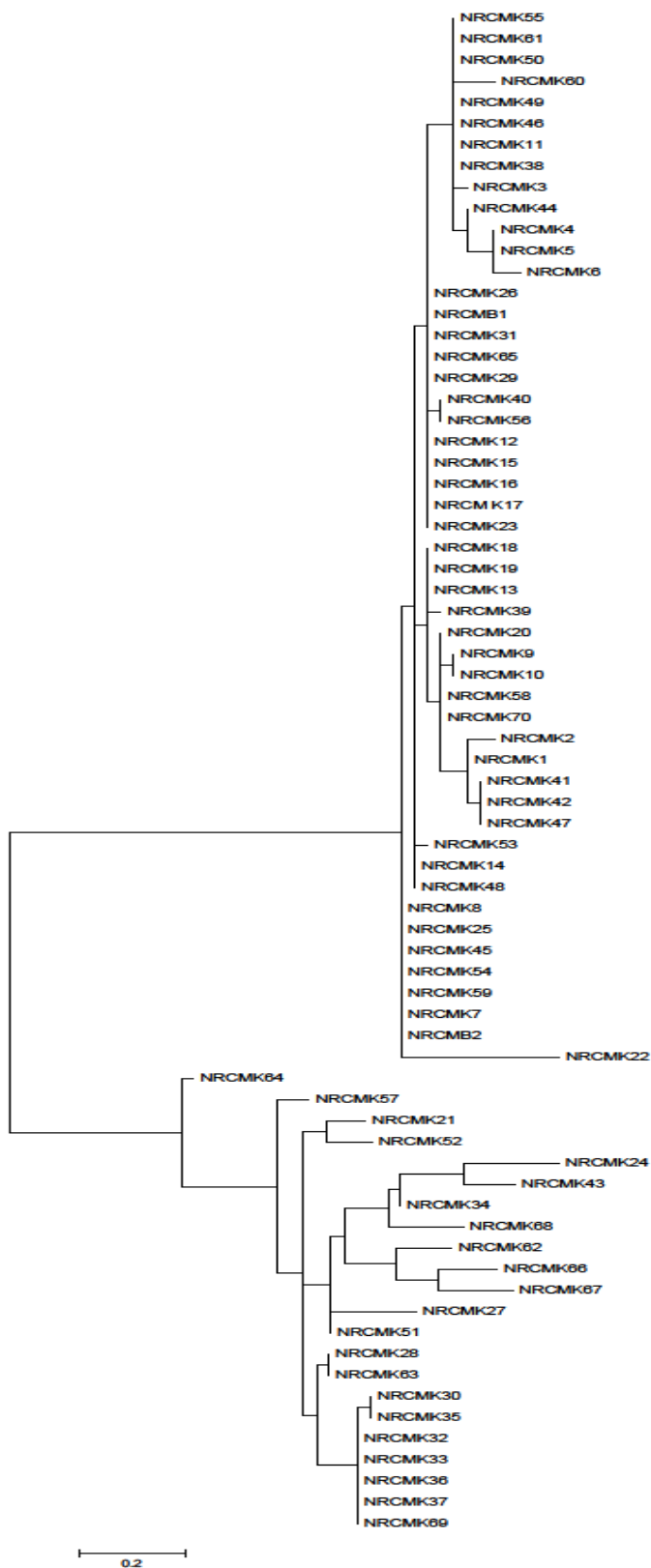
NRCM K42	KC020722	Uncultured rumen bacterium clone	HM816731	97
NRCMK49	KC020723	Butyrivibrio species	EU714408	98
NRCM K56	KC020724	Uncultured rumen bacterium clone	FJ032385	95
NRCM K48	KC020725	Uncultured rumen bacterium clone	JX003956	93
NRCM K47	KC020726	Streptococcus sp.	GQ139522	99
NRCM K50	KC020727	Butyrivibrio species	AM039823	99
NRCM K59	KC020728	Uncultured rumen bacterium clone	AB270006	94
NRCM K41	KC020729	Uncultured organism clone	HQ746244	97
NRCM K60	KC171704	PseudoButyrivibrio ruminis	NR026315	97
NRCM K61	KC171705	Butyrivibrio sp.	EU714408	98
NRCM K62	KC171707	Succinivibrio dextrinosolvens	NR026476	99
NRCM k63	KC171716	Prevotella ruminicola	AB501169	94
NRCM K64	-	Uncultured rumen bacterium clone	GU302745	96
NRCM k65	KC171713	Bacteroidetes bacterium clone	HM104836	98
NRCM K66	KC171715	B.fibrisolvens	X89979	99
NRCM k67	KC171709	Sporanaerobacter acetigenes	NR025151	96
NRCM K43	KC171712	Uncultured rumen bacterium clone	AB270037	98
NRCM K54	KC171714	Uncultured rumen bacterium clone	AB270090	97
NRCM K57	KC171708	Prevotella species	JF893676	90
NRCM K68	KC171710	Uncultured rumen bacterium clone	DQ673497	92
NRCM K69	KC171706	Prevotella species	AB003385	96
NRCM K70	KC171711	Ruminococcus flavefaciens	AM915269	98
NRCM B1	JQ290364	Prevotella species clone	AB501166	93
NRCM B2	JQ290365	Uncultured rumen bacterium clone	AB244120	98

together in three subgroups. Sub-group I contained two uncultured rumen bacteria. Sub-group II contained nine clones out of which six are of uncultured rumen bacteria and another three are of *Prevotella ruminicola* (NRCMK24), *Succinivibrio dextrinosolvens* (NRCMK62) and *Butyrivibrio fibrisolvens* (NRCMK66). Sub-group III, contained 9 clones, out of which, eight clones are of *Prevotella* species (NRCMK30, NRCMK32, NRCMK33, NRCMK35, NRCMK36, NRCMK37, NRCMK63, NRCMK69) and one is of uncultured rumen bacteria.

In this experiment, majority of bacteria were found to be of *Prevotella* species as they take part in digestion of feed stuffs in the rumen. *P. ruminicola* is a proteolytic bacterium and plays a key role in ruminal protein degradation (Wallace, 1996; McKain et al., 1992). Whitford et al. (1998) also reported that 16S rDNA sequences similar to those of *P. ruminicola* prevailed in isolated material from domestic cattle. Pandya et al. (2010) while studying the bacterial diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), reported that, the CFB (Cytophaga-Flavobacteria-Bacteroides) bacteria were less numerous than Firmicutes in the rumen of buffaloes. This suggests that, the particular type of diet given to the animals can have a significant impact on the bacterial diversity of the rumen (Hungate, 1969). The

microbial population in the mithun (*Bos frontalis*) in NE region of India fed on mixed tree leaves and rice straw based diet are different from other domestic animals.

The clones of fibrolytic bacteria like *B. fibrisolvens* and *R. flavefaciens* were isolated (Khampa et al., 2006; Leng et al., 2011), but *Fibrobacter succinogens* was not isolated similar to the findings of Deng et al. (2007). The starch degrading bacteria (*Succinivibrio dextrinosolvens*) were also isolated in the present study. *Streptococcus* species were isolated in the present experiments which are responsible for starch degradation (Cotta, 1988) and some strains of *Streptococcus* are also responsible for fibre degradation. Deng et al. (2007) found that the numbers of cellulolytic and amylolytic bacteria were increased in mithun as compared to cattle (*Bos taurus*) and dominant bacteria isolated in the study of Leng et al. (2011) were cellulolytic and amylolytic. In our study also, both cellulolytic and amylolytic bacteria were isolated from the rumen of mithun. Sulphate reducing bacteria (*Sporanaerobacter acetigenes*) was isolated in the present study. Though many bacteria reduce sulphate during their synthesis of sulphur containing amino acids, presence of sulphate reducing bacteria in rumen of mithun is benefit for growth of the animal.



**Figure 3.** Phylogenetic tree of 16S rDNA sequences of clones recovered from rumen of mithun.

## Conclusion

The experiment showed that, feeding of mixed tree leaves and rice straw based diet harbor diversified species of bacteria, that is, *P. ruminicola*, *B. fibrisolvens*, *P. ruminis*, *S. dextrinosolvens*, *R. flavefaciens*, *Streptococcus* species etc. in the rumen of mithun for digestion of feed stuffs.

## Conflict of Interests

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

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Review

## Biofilm in aquaculture production

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A biofilm is an assemblage of microbial cells which is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. It may form on a wide variety of surfaces, including living tissues, medical devices, industrial or potable water system pipe or natural aquatic systems. A well-diversified organism such as algae, bacteria, protozoa, arthropods, etc. may be observed in the biofilm assemblage. The biofilm structure depends on the nature of substratum, hydrodynamics of system, nutrient availability, light and grazing capacity of organism. It has been observed that the introduction of substrata for the development of biofilm in the aquaculture system play a significant role. Biofilm organisms are microscopic and highly nutritious. The organisms of biofilm may serve as single cell protein and are easily harvested by all size of cultured species in aquaculture as compared to planktonic organism in the water column. Biofilms are considered as good quality protein source (23-30%). Microalgae and heterotrophic bacteria are rich source of immune enhancers, growth promoters, bioactive compounds and dietary stimulants which can enhance growth performance of cultured organism. Substrata minimize the mortality by providing shelter and hiding places to cultured organisms. The attached nitrifying bacteria contained in biofilm improve the water quality by lowering ammonia waste from culture system through nitrification process. Biofilm based low cost technology will help resource poor farmers in generating protein rich nutrient in sustainable manner from aquaculture. An attempt has been made to review the role of biofilm in aquaculture.

**Key words:** Biofilm, fish growth, sustainable, water quality, survival, nutrient quality.

### INTRODUCTION

Aquaculture has evolved on a large scale during the last two decades and has become a major source of protein for a large population of the world. The current worldwide growth rate of the aquaculture (8.9–9.1% per year since 1970s) is needed in order to cope with short supply of protein rich food, particularly in the developing countries (Gutierrez-Wing and Malone, 2006; Matos et al., 2006; Subasinghe, 2005). Aquaculture has emerged as one of

the important branches of food production. Sustained productivity along with enhanced production is the major goal of aquaculture. Expansion of pond area and intensive farming in the pre-existing ponds has great potential for further development of aquaculture throughout the world. However, high stocking densities and frequent use of water, feeds and fertilizers in aquaculture intensification, leads to increased waste

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production (Beveridge et al., 1997). On the other hand, operation of intensive aquaculture also demands huge investment and technical expertise, which are not affordable by resource-poor farmers. However, environmental considerations and economic conditions of poor farmers are the major limitations in the growth of aquaculture. Especially, intensive aquaculture coincides with the pollution of the water by an excess of organic materials and nutrients that are likely to cause acute toxic effects and long term environmental risks (Piedrahita, 2003) by increasing waste production (Beveridge et al., 1997). To keep dissolved nutrients at low level, large amount of water must be exchanged, increasing the costs of production in aquaculture. An alternative way to maintain high water quality is biological treatment such as use of filters with a high surface-volume ratio, pre-colonized by microorganisms that absorb excess nutrients from the water (Wheaton, 1977). The relatively new alternatives to previous approaches are the application of bio-flocs and biofilm in aquaculture (Avnimelech, 2006). Biofilms - a microbial consortium associated with a matrix of extracellular polymeric substances bound to any submerged surfaces are responsible for many biogeochemical cycles in aquatic ecosystems, especially nitrogen cycling (Decho, 1990; Meyer-Reil, 1994). The major driving force is the intensive growth of heterotrophic bacteria to reduce the overload of unwanted components in the aquaculture. The presence of biofilms in aquaculture reduces the cost of shrimp production by minimizing water exchange (Thompson et al., 2002). It was also demonstrated in several studies that in the presence of biofilm, reduction, or even suppression of water exchange, did not cause any damage to the cultured organisms faced due to stress (Hopkins et al., 1995; McIntosh, 2000).

Thompson et al. (2002) indicated that nitrogen uptake by a biofilm may help to reduce the occurrence of pathogenic bacteria in culture system, as these pathogenic microorganisms normally occur in situations where nitrogenous compounds reach extremely high values (Austin and Austin, 1999; Brock and Main, 1994). Moreover, many microalgae present in biofilms are able to produce antibiotics that prevent pathogenic bacterial growth (Austin and Day, 1990; Alabiet et al., 1999). Inhabiting protozoa in biofilms can also control the abundance of pathogenic bacteria through grazing (Thompson et al., 1999). Thus, it is possible that biofilm removal can increase the risk of developing pathogenic bacteria (Thompson et al., 2002).

The manipulation of C:N ratio by use of biofilm on substrata in freshwater finfish and prawn production in extensive pond enhances aquaculture production (van Dam et al., 2002; Azim and Little, 2006; Hargreaves, 2006). The development of biofilm requires installation of hard substrata or application of cheap carbohydrates resources which could potentially be produced within the farmer's traditional agricultural systems (Asaduzzaman et al., 2008). It has been observed that both survival and growth of freshwater prawn were significantly higher due

to provision of substratum as compared to traditional production system without substratum (Cohen et al., 1983; Tidwell and Bratvold, 2005; Uddin et al., 2006). The biofilm acts as additional natural food, shelter to minimize territorial effects, improves water quality through trapping of suspended solids, organic matter breakdown, enhances nitrification through the microbial activity and heterotrophic production of single cell protein (McIntosh, 2000; Hari et al., 2004; Crab et al., 2007; Avnimelech, 2007). The main objective of this review is to assess the effect of biofilm in enhancing aquaculture production in a sustainable manner.

## BIOFILM FORMATION IN AQUATIC ENVIRONMENT

Biofilms may form on a wide variety of surfaces, including living tissues, medical devices, industrial or potable water system pipe or natural aquatic systems (Donlan, 2002; Kordmahaleh and Shalke, 2013). Biofilm formation begins with the accumulation of organic molecules on any submerged surface. Few hours after the establishment of the macromolecular film, bacterial colonization begins (Whal, 1989). The main advantage of biofilm formation is the protection of the organisms from the effects of an adverse environment. A multispecies microbial culture can provide and maintain appropriate physical and chemical environments for further growth and survival (King et al., 2008).

Biofilm formation is a dynamic process, capable of responding to environmental stimuli. Initially, cells organize themselves into micro-colonies, followed by division and cell recruitment, they grow and encase themselves in an extracellular matrix. Within this matrix, complex and differentiated associations can be formed, which facilitate nutrient uptake (Hall-Stoodley et al., 2004; Toutain et al., 2004). The redistribution of attached cells by surface motility is one of the most important mechanisms of biofilm formation (Hall-Stoodley and Stoodley, 2002). The development of biofilm depends on constituents of the organism(s), the properties of the surface being colonized and the physico-chemical conditions of the aqueous environment. Detachment from biofilms can be caused by a number of factors including external perturbations or internal processes, though many species appear to use dispersal as an active means of colonizing new niches (Sauer et al., 2002; Hall-Stoodley et al., 2004). Biofilm formation is generally thought to proceed as follows: (1) individuals colonize a surface, (2) individuals form micro-colonies and (3) microcolonies form biofilms (Johnson, 2008).

### Nutrient availability in aquaculture ecosystem

Nutrients supply energy for the growth and development of heterotrophic bacterial population. Pradeep et al. (2004) reported increase in aerobic heterotrophic bacte-

rial populations in water over due course of time and reached at peak during the 4<sup>th</sup> week, followed by subsequent decrease, attributing to the role of nutrients availability. Initially, higher levels of nutrients lead to bacterial multiplication and with reduction in nutrient levels, the population starts declining. The change in the diversity of bacterial community strongly depends on temporal change in the quality and quantity of organic substrata available (Gatune et al., 2012). Inorganic nutrients have a strong effect on periphyton biomass (Aizaki and Sakamoto, 1988; Lohman et al., 1992; Ghosh and Gaur, 1994). Mattila and Raesaenen (1998) found that periphyton biomass and productivity can be used as an indicator of eutrophication in natural waters. In the most freshwater studies, phosphorous was identified as the limiting nutrient (Ghosh and Gaur, 1994; Vymazal et al., 1994), but sometimes nitrogen (Barnese and Schelske, 1994) and carbon (Sherman and Fairchild, 1989) may also act as limiting factors. Aquaculture ponds are fertilized with phosphorus and nitrogen, which serves as important source of nutrients for biofilms as well.

Action of nitrogen and carbon, as limiting factor, depends on the algal species and on other environmental factors such as hardness and acidity. High silicon: phosphorus (Si:P) and nitrogen: phosphorus (N:P) ratios favoured diatoms while low N:P and Si:P ratios favoured cyanophytes in a reservoir in Patagonia (Baffico and Pedroso, 1996). Similarly, high Si:N or Si:P ratios favoured diatoms but low N:P ratios favoured cyanophytes and high N:P ratios favoured chlorophytes in periphyton of the Baltic Sea (Sommer, 1996). Dissolved organic matter may play a role in determining the structure of the biofilm. Biofilm communities treated experimentally with dissolved organic carbon contained less mucilage than untreated controls (Wetzel et al., 1997). Molobela et al. (2010) reported that nutrients boost the biofilm cells growing in the medium which enhance extracellular polymeric substance (EPS) production.

### Effect of grazing

Grazing is one of the most important determinants of biofilm biomass. Bacterial abundance is controlled by the grazing exerted by flagellates and ciliates (Thompson et al., 2002). Grazing has an overriding effect on biomass while the effect of nutrients has much less apparent effect due to the ability of the periphyton to recycle and utilize nutrients from the substratum (Hill et al., 1992; Steinman et al., 1992; Pan and Lowe, 1994). On plastic substrates in tilapia cages in Bangladesh, filamentous Chlorophyceae and Myxophyceae dominated the periphyton before fish stocking, whereas after stocking of the fish, diatoms became more abundant (Huchette et al., 2000), indicating the effect of grazing. Biofilms provide easy availability of food to the cultured organisms and thus help in enhancement of fish production.

### Suitable species for biofilm-based aquaculture

It has been noticed that consumption pattern and feeding efficiency on biofilm depend on the grazing efficacy of cultured species. However, fishes are found to be fast grazers than crustaceans (Asaduzzaman et al., 2010). Biofilm has already been considered an important food source for Nile tilapia (Shrestha and Knud-Hansen, 1994) and carp (Ramesh et al., 1999). Azim et al. (2001) demonstrated that rohu (*Labeorohita*) and orange-fin labeo (*Moruliuscalbasu*) are more suitable candidates for periphyton based aquaculture than kurialabeo (*L. gonius*). In penaeid shrimp culture, consumption of periphyton developed over submerged substrate significantly improved growth of penaeid shrimp, *Fenneropenaeus paulensis* (Ballester et al., 2007; Thompson et al., 2002), *Penaeusesculentus* (Burford et al., 2004), *L. vannamei* (Audelo-Naranjo et al., 2011; Moss and Moss, 2004) and *P. monodon* (Anand et al., 2012; Khatoun et al., 2007). The culture performance of the freshwater prawn *Macrobrachium rosenbergii* improves in the presence of artificial substrata (Tidwell et al., 1998). Similar grazing activity was reported by Erler et al. (2004) who found that *Farfantepenaeus merguensis* grazed on epibiota on AquaMats® and significantly reduced the attached biomass. *P. monodon* grown over periphytic microalgae attained significant improvement in body weight compared to the control (Anand et al., 2012; Arnold et al., 2009). Bourne et al. (2006) applied biofilm within a larval rearing tank of the tropical rock lobster (*Panulirusornatus*). Biofilm can be used to promote the growth of bottom feeder fishes like *Cirrhinusmrigala* (Bharti et al., 2013; Mridula et al., 2006). The biofilm was considered a good tool during *F. brasiliensis* nursery phase, mainly due to enhancement of survival through maintenance of water quality (Viau et al., 2013).

### Enhancement of production

Shrimp showed a higher final weight in the tanks with biofilm, leading to higher biomass at the end of the experiments (Thompson et al., 2002). Ramesh et al. (1999) observed that easily biodegradable sugarcane bagasse, having more fibre and surface area favoured better growth of fish through bacterial biofilm than paddy straw and *Eichhornea*. The growth of rohu (*L. rohita*) in the presence of sugarcane bagasse, paddy straw and dried *Eichhornea* for settlement of biofilm, was higher by 47.5, 29.1 and 17.6%, respectively than the control. Similarly, they observed the growth of common carp (*Cyprinuscarpio*) in the presence of substrata like sugarcane bagasse, paddy straw and dried *Eichhornea* was higher by 47.4, 32.9 and 20.7%, respectively than the control. Umesh et al. (1999) conducted an experiment with sugarcane bagasse as substratum and found that the growth of fish was remarkably high in the treatments



with an average of 50% over the controls. Natural biodegradable substrata like paddy straw and sugarcane bagasse favoured better growth of the fish than non-biodegradable substrata like plastic sheet and tile, with paddy straw turning out to be the best substratum for aquaculture of *C. mrigala* (Bharti et al., 2013). Keshavanath et al. (2012) evaluated the culture of rohu and common carp in the presence of periphyton developed on different types of substrata namely sugarcane bagasse, palm leaf, coconut leaf and bamboo mat in poultry-manured ponds and found that all the four substrata induced significantly high growth, production and survival rate in both fish compared to substrate free culture system, indicating the importance of biofilm in aquaculture. The growth of rohu under sugarcane bagasse, palm leaf, coconut leaf and bamboo mat treatment was 93.69, 103 and 44% higher, respectively than that of the control, while common carp performed 98, 74, 100 and 20% better than the control in the same treatments, respectively.

Schweitzer et al. (2013) observed that the final biomass of *L. vannamei* was 31.4% more and the survival rate was also significantly high in the tank with substrata. Asaduzzaman et al. (2008) found that the addition of biofilm on substrata increased net yield of prawn from 370 to 456 kg ha<sup>-1</sup> 120 day<sup>-1</sup> that is 23% higher yield than absence of biofilm.

Azim et al. (2002a) found the highest net yield of 2098 kg ha<sup>-1</sup> in bamboo treatment, followed in diminishing order by 2048 kg ha<sup>-1</sup> (jutestick), 2032 kg ha<sup>-1</sup> (kanchi), 1960 kg ha<sup>-1</sup> (feed) and 1226 kg ha<sup>-1</sup> (control). Azim et al. (2001) and Keshavanath et al. (2001) have shown that the use of various substrata contributed to the growth and production of different aquaculture species in freshwater ponds through the development of biofilm on the substrata. Tidwell et al. (1998) reported that adding substrate in freshwater ponds increased prawn production by 20% as well as average size by 23%. Easy availability of food through biofilm as well as its positive impact on water quality helps in attaining better growth and higher production of finfish and shellfish.

### Improvement in water quality

Biological nitrification can be accomplished in two types of systems like suspended and attached growth. Under a suspended growth environment, the microorganisms are freely mobile in the liquid media being in direct contact with the bulk water. In an attached growth system, on the other hand, microorganisms are grown in a viscoelastic layer of biofilm that are attached on the surface of a solid support medium. Thus, this process is also called a fixed film process in which the individual bacteria are immobilized. Attached growth on a fixed biofilm system offers several advantages when compared to suspended growth processes, such as handling convenience, increasing process stability in terms of withstanding shock

loading and preventing the bacterial population from being washed off (Fitch et al., 1998; Nogueira et al., 1998). Pradeep et al. (2003b) observed improved water quality with application of probiotic and biofilm during the culture of fingerlings of *Catla catla*. Lower level of total ammonia concentrations in the substrata based treatments was recorded compared to feed and control treatments. This might be due to higher nitrification rates in substrata treatments (Azim et al., 2002a).

Several researchers have demonstrated that the presence of nitrifying bacteria in the biofilm decreased ammonium level in the cultured water (Bharti et al., 2013; Langis et al., 1988; Ramesh et al., 1999). In fact, decrease in ammonium and parallel augmentation in nitrite and nitrate concentrations in the experiment indicates that nitrifying bacteria present in the biofilm play a significant role in water quality management (Kaiser and Wheaton, 1983). On the other hand, ammonium is also absorbed by the microalgae that use this element to produce new biomass (Thompson et al., 2002). Nitrogen uptake by a biofilm may help to reduce the occurrence of pathogenic bacteria, since these microorganisms normally occur in situations where nitrogenous compounds reach extremely high values (Austin and Austin, 1999; Brock and Main, 1994). The direct discharge of large amount of waste water from hatcheries may cause eutrophication in rivers and coastal waters, but use of different periphytic microalgae in aquaculture can significantly reduce ammonia and nitrite levels from the system (Ziemann et al., 1992). In *Oscillatoria* tanks, total ammonia nitrogen (TAN), NO<sub>2</sub>-N and soluble reactive phosphorus (SRP) levels were reduced more than 80% against approximately 60% in *Amphora*, *Cymbella*, and *Navicula* tanks in spite of no water exchange during the 16-day culture period.

The use of biofilm to reduce excess nutrients in hatchery tanks does not only maintain the water quality, but also reduces the risk of pathogen introduction since the system does not require water exchange (Khathoon et al., 2007). They observed that the water was very clear in the experimental tanks even without water exchange compared to the control tanks which was turbid. Biofilm can reduce phosphorous (Bratvold and Browdy, 2001; Hansson, 1989) and other nutrients in the water. In addition, biofilm communities reduce water turbidity by trapping organic matter in the column (van Dam et al., 2002). Anand et al. (2013) used bamboo as substratum for the culture of *P. monodon* and found the water quality parameters across the treatments were within desired range and were optimum for growth of cultured shrimp. The low concentration of nitrite observed during the culture period with practice of raceway system indicates the complete oxidation of ammonia to nitrate (Cohen et al., 2005). The low concentration of ammonia and nitrite in the aquaculture without exchange of water is accomplished by microbial activities which remove these compounds through nitrification process (Ebeling et al., 2006).

The addition of substrate in freshwater fish ponds improved water quality by lowering total ammonia concentration through the biofilm formation on substratum (Dharmaraj et al., 2002; Ramesh et al., 1999). Azim et al. (2002b) reported that the average total ammonia concentration in substratum-based freshwater aquaculture ponds ( $0.56 \text{ mg L}^{-1}$ ) was significantly lower than the substratum-free ponds ( $0.95 \text{ mg L}^{-1}$ ) and biofilm on substratum reduces the nitrite-nitrogen concentration of the water column as well (Asaduzzaman et al., 2008). Natural and biodegradable substratum in aquaculture provides favourable water quality parameters for the culture of carp (Keshavanath et al., 2012). The nitrite concentration was significantly high in the feed treatment compared with biofilm (Viau et al., 2013).

### Biofilm as food source

Many trials in fish culture ponds have demonstrated the utility of submerged substratum in enhancing fish production (Azim et al., 2005; Asaduzzaman et al., 2008; Jana et al., 2004; Keshavanath et al., 2002). Microalgae and probiotic bacterial products are well-known for their nutritional benefits (Ju et al., 2009) and widely used as dietary stimulants to shrimp juvenile (Ju et al., 2009; Wang, 2007). Burford et al. (2004) showed that epiphytes contributed substantially (39–53%) to shrimp requirements of carbon and nitrogen. Azim and Wahab (2005) reported that in substratum-based freshwater fish ponds, periphyton served as an additional food source. Khatoon et al. (2007) observed that the specific growth rate of shrimp post-larvae increased 28% in the presence of substratum. Ballester et al. (2003) determined that growth and survival of *F. paulensis* post-larvae did not enhance in the presence of artificial substrata that had their biofilm periodically removed, indicating the importance of biofilm as food. Therefore, though there may be a synergism of physical and biological aspects related to the use of artificial substrata, it seems that the nutritional role of the biofilm is most likely the important aspect affecting the culture of *F. paulensis* post-larvae in cages. The biofilm formed on the substrata is composed of organisms that belong to the natural diet of penaeid shrimp and serve an additional source of nutrition for the post-larvae having a high quality diet (Ballester et al., 2007).

Feeding with substrate based biofilm had a significant effect on the production of *Tor khudree* and *L. fimbriatus*, as net production that was 30–59% greater than that of fish in tanks without substratum (Keshavanath et al., 2002). The growth response of *Heteropneustes fossilis*, in the presence of sugarcane bagasse as a substratum, was observed to be high in comparison to absence of substratum (Radhakrishnan and Sugumaran, 2010). Similarly, Bratvold and Browdy (2001) reported high shrimp production and low feed conversion ratio (FCR) during the culture of *L. vannamei* in a high density culture

system with artificial substrata (Aquamats™). They also reported that artificial substrata increased the nitrification in the tanks, which resulted in decreased concentrations of  $\text{NH}_3$  nitrogen. Moss and Moss (2004) reported enhancement in the production of *L. vannamei* post-larvae stocked at different densities in a flow-through system provided with Aquamats™. The authors suggested that increased shrimp growth in the presence of substrata was due to the availability of attached particulate organic matter and that the use of artificial substrata could lessen the negative effects of high stocking density during the nursery phase.

### Nutritive quality of biofilm

It has been documented that nutritional composition of biofilm can be broadly considered as appropriate to fish dietary needs (Azim et al., 2002a; Dempster et al., 1993; Makarevich et al., 1993). Proximate composition of biofilm varied from 23-30% for protein, 2-9% for lipid, 25-28% for NFE and 16-42% for ash (Azim et al., 2005; Thompson et al., 2002; van Dam et al., 2002). Most fish farmers use complete diets comprising protein (18-50%), lipid (10-25%), carbohydrate (15-20%), ash (<8.5%), phosphorus (<1.5%), water (<10%), and trace amounts of vitamins and minerals (Craig and Helfrich, 2002). This indicates that nutritional quality of biofilm can be used as dietary supplement in the culture of fish and shrimps. *P. monodon* juvenile needs 35 to 40% protein (Alava and Lim, 1983; Shiao, 1998) and up to 10% lipid (Akiyama et al., 1992). The optimum requirement of protein for Indian major carps is 30% (Renukaradhya and Varghese, 1986). The protein supplementation has been observed by the microbial communities of biofilm (Burford et al., 2004; Wasielesky et al., 2006).

Biofilms are considered as good quality protein source (Oser, 1959). Therefore, biofilm attributes better growth in fish as well as shrimps (Anand et al., 2013). Apart from being a source of macronutrients, microalgae and heterotrophic bacteria are rich source of immune enhancers (Supamattaya et al., 2005), growth promoters (Kuhn et al., 2010), bioactive compounds (Ju et al., 2008) and dietary stimulants (Xu et al., 2012) which can enhance growth performance of cultured shrimp. Hence, it can be inferred that these beneficial effects of algae and microbes in biofilm might have attributed to improved growth response in tiger shrimp juvenile. Fish and shrimp larvae are very sensitive to the deficiency of certain fatty acids (FA) such as the n-3 poly unsaturated fatty acids (PUFA) (Sorgeloos and Lavens, 2000; Watanabe et al., 1983). This essential nutrient is ultimately derived from the natural food sources such as the phytoplankton, zooplankton and macro-invertebrates (Parrish, 2009). Even bacteria are abundant in the natural food sources and available as a potential food source for cultured species (Azim and Wahab, 2005; Burford et al., 2004;

Keshavanath and Gangadhar, 2005). Various studies have suggested that bacteria acted as an important nutritional source for penaeid shrimp in promoting grazing ability, growth and survival when occurring as biofilm on structures in semi-intensive and extensive ponds (Azim and Wahab, 2005; Bratvold and Browdy, 2001; Keshavanath and Gangadhar, 2005). The presence of protozoans and nematodes in the biofilm is probably an important nutritional increment for shrimp growth (Ballester et al., 2007). These organisms have a higher protein to energy ratio and, due to their ability to synthesize long chain polyunsaturated fatty acids, they enrich the quality of microbial aggregates, such as the biofilm (Zhukova and Kharlamenko, 1999). Lipid contents were high in periphyton derived from bamboo and kanchi and low in jutestick substrata. The ash content was high in periphyton from jutestick, followed by bamboo and kanchi. The energy content of periphyton was similar in bamboo and kanchi treatments and higher than jutestick treatment, while bottom sediments contained 93–95% ash and negligible amounts of protein and lipid (Azim et al., 2002).

#### Autotrophic and heterotrophic biomass in biofilm

Herbivore food chain reduces the loss of energy during transfer from one trophic level to another. Therefore, selection of herbivore fish and enhancement of autotrophic biomass is one of the best strategies to boost the fish production in aquaculture by reducing the cost of production. Biofilm serves as a good source of autotrophic and heterotrophic biomass because several types of phytoplankton as well as zooplankton are attached with it. The harvesting of energy from the attached biomass by fish and shrimp is easier as compared to planktonic form. Qualitative analysis of biofilm by Anand et al. (2013) indicated that the harvested biofilm was composed of 37 genera of algae belonging to Bacillariophyceae (13 genera), Cyanophyceae (10), Chlorophyceae (11) and Euglenophyceae (3) and 5 genera of zooplankton belonging to Rotifer (3) and Crustacea (2). Viau et al. (2013) reported that the chlorophyll-a concentration in water was higher in biofilm and feed based treatment ( $1.36 \mu\text{g L}^{-1}$ ) than the feed treatment ( $0.49 \mu\text{g L}^{-1}$ ) alone. The biofilm bacterial density on substratum per unit weight was 100 times more than the water (Ramesh et al., 1999). Increasing the C:N ratio raised the total heterotrophic bacterial population in the water column, sediment and periphyton (Asaduzzaman et al., 2008). Karunasagar et al. (1996) found the highest cell density on the plastic surface followed by cement slab and steel surface. On contrast to this, King et al. (2008) used six different types of substrata for growing of biofilm in recirculating aquaculture. Even, after three days of introduction of substrata, they reported the numbers of bacteria remained constant throughout the experiment and at the same time they found that there was no significant difference in bacterial count on these different substrata.

#### Substrata enhance survival rate

Abdussamad and Thampy (1994) observed high levels of shrimp damage in high density rearing systems due to cannibalism and reported increased chance of cannibalism in the case of newly moulted specimens. The introduction of substrata in the culture system is one of the ideal methods to increase the survival through minimizing the cannibalism. Substrata, apart from providing biofilm, provide shelter and protection from predators. This alone is an interesting feature since several studies have showed an inverse relation between stocking density and shrimp growth (Martin et al., 1998; Preto et al., 2005; Wasielesky et al., 2001). Khattoon et al. (2007) showed the presence of post-larvae inside the PVC coated pipes which could have served as refugium to the moulting post-larvae leading to enhanced survival as a significantly high survival (51–60%) was found compared to the control (37%). Sandifer et al. (1987) reported high survival (24%) in the nursery rearing of *L. vannamei* in the tanks where fiberglass window screens were provided. In addition to increasing food supply, the presence of substratum appears to reduce stress by acting as a shelter or hiding place for fish (Keshavanath et al., 2002). Ju et al. (2009) recorded significantly high growth and survival in *L. vannamei* with diet supplemented with microalgae.

Ballester et al. (2007) reported that shrimp *F. paulensis* post-larvae reared in cages had a considerable increase in their biomass and survival with the addition of polyethylene substratum. During the experimental period, it was observed that shrimps were constantly occupying the substratum and feeding on the biofilm. Besides, the nutritional contribution provided by the biofilm, the physical presence of artificial substratum within the culture units promotes the enlargement of the area for shrimp distribution (Ballester et al., 2007). Bratvold and Browdy (2001) observed that the presence of artificial substratum delayed the negative effects of overcrowding, contributing to a better performance of *L. vannamei* reared in an intensive culture system. Furthermore, Tidwell et al. (1998) were able to improve the culture performance of the freshwater prawn *M. rosenbergii* as they utilized artificial substrata to reduce the negative effects derived from the strong territorial behaviour of this species.

Addition of periphyton substrata increased the survival of prawn 63 to 72% as compared to periphyton free treatment (Asaduzzaman et al., 2008). Survival of shrimp (*F. brasiliensis*) maintained in the biofilm was significantly higher than that observed in the feed treatment (Viau et al., 2013).

#### Use in vaccination

Natural resistant property of bacterial biofilms for development of effective oral vaccines is simple and cheap approach. Azad et al. (1999) reported that the oral route

of vaccination is one of the simple, cheap and ideal techniques among the various methods of vaccination for mass administration to fish of all sizes. Oral vaccination is regarded beneficial in aquaculture as it is non-stressing and accessible to fish of any size, age and numbers (Quentel et al., 1997).

However, direct administration of antigen during the oral vaccination showed poor response due to digestive degradation of antigens in the fore-gut before the vaccine reached immune-responsive areas in the hind-gut and other lymphoid organs (Johnson and Amend, 1983; Rombout et al., 1985). Strategies developed for improvements of oral vaccination have attempted to avoid this gastric destruction especially by the use of encapsulated antigen microspheres (Dalmo et al., 1995; Piganelli et al., 1994).

Azad et al. (1997) developed and evaluated a biofilm of *A. hydrophila* for oral vaccination of carp which induced significantly higher antibody titres and protection compared to a free cell vaccine. Bacterial biofilm developed on substrata have been found to be resistant to antibiotics (Anwar and Costerton, 1990), phagocytosis and the killing effect of whole blood and serum (Anwar et al., 1992) due to presence of a protective layer of glycocalyx.

The glycocalyx of biofilm is a polymer of neutral hexoses which encapsulates and possibly protects the bacterial surface antigens even from digestion in the gut (Costerton and Irvin, 1981).

This property of biofilm vaccine is reported to facilitate longer retention of antigens in the gut and lymphoid tissue and hence, might have resulted in the early and heightened primary antibody response (Azad et al., 2000). The importance of biofilm vaccine has more relevance for oral vaccination of carnivorous fish where stomach is well developed in the digestive system (Nayak et al., 2004).

*Bacillus* spp. are prominent bacteria in biofilm (Pradeep et al., 2004) and *B. subtilis* has been shown to possess antitumor and immunomodulatory activities in fish (Cohen et al., 2003). Some studies have demonstrated that *B. subtilis* and spores of *B. subtilis* act as probiotics since they promote growth and viability of the beneficial lactic acid bacteria in the intestinal tracts of humans and some animals (Hoa et al., 2000).

Alya et al. (2008) showed that *B. subtilis* and *Lactobacillus acidophilus* inhibited the growth of *A. hydrophila* in the *O. niloticus*. The *B. subtilis* inhibited the establishment of *P. fluorescens* in the *O. niloticus*. The two *Bacillus* strains, *B. subtilis* 2335 and *B. licheniformis* 2336 are well characterised and a number of clinical studies have been used to demonstrate probiotic effects (Bilev, 2002).

## CONCLUSION

Improved water quality and nutrient availability through biofilm enhance the survival rate and growth of fish in the substrate based aquaculture. Heterotrophic bacterial load is very high in biofilm in comparison to water indicating

vast scope for biofilm utilization in production of fish and shellfish in aquaculture. Biofilm based microorganisms enhance the immune response of aquaculture species through oral vaccination. Locally available cheap natural biomass may be used as substratum for biofilm formation in aquaculture so as to convert them into a valuable resource within a pond ecosystem with a view to promote sustainable aquaculture especially in developing countries.

## Conflict of Interests

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

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Full Length Research Paper

# Effectiveness of *Myrothecium roridum* for controlling water hyacinth and species identification based on molecular data

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*Myrothecium roridum* isolates were evaluated for their effectiveness in controlling the aggressive water hyacinth and molecular identification was conducted using internal transcribed spacer (ITS) rDNA region and amplified fragment length polymorphism (AFLP) markers. *M. roridum* isolates were collected from nine provinces of Thailand. Seventy isolates were included in the primary screening, using colonial growth rate and spore number measurement. The results indicate that five isolates were the most aggressive and so were selected to evaluate their effectiveness in controlling the water hyacinth. The result showed that *M. roridum* isolate KKFC 408 had significantly highest disease severity ( $P < 0.05$ ) and the highest reduction for the fresh weight of water hyacinth. These five isolates DNA were amplified and sequenced using ITS1 and ITS4 universal primer for species identification. The data analyzed showed that five sequences of *M. roridum* were in the same group agreeing with other sequences of *M. roridum* recorded in the GenBank database, which correlated to morphological observation results. The AFLP result indicated that fifteen isolates of *M. roridum* were divided into four subgroups which were not correlated to the geographical area.

**Key words:** Amplified fragment length polymorphism (AFLP), internal transcribed spacer (ITS) of rDNA region, mycoherbicide.

## INTRODUCTION

Water hyacinth (*Eichhorniacrassipes* (Mart.) Solms) is a water plant belonging to the family Pontederiaceae. This aquatic weed is counted as a major problem due to its rapid productivity and the difficulty of eradication. Water hyacinth affects irrigation, water flow, water use and navigation. The problems caused by water hyacinth

include obstructing waterways, impeding drainage, destroying wildlife resources, reducing outdoor recreation opportunities, lowering dissolved oxygen levels resulting in reduced available oxygen for animals and other plants and a health risk by enabling the breeding of mosquitoes, bilharzias and other human parasites (Okunowo et al.,

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2013). Each year, the government spends several millions baht removing this species from water resources. Control and removal can be by either mechanical or chemical methods. However, both methods have demonstrated that they do not remove this aquatic weed effectively. Consequently, a biological controlling method is a plausible alternative; this could reduce expense and would not be toxic to the environment. However, there is the disadvantage of biological method that takes time for working and controlling. In Thailand, biological control of the water hyacinth is still limited, although there is a wide range of research at the international level, especially in using asexual Ascomycete fungi to control the water hyacinth or other aquatic weeds. A pathogenic fungi may have many advantages as: (i) most pathogens of plants are fungi (ii) they are destructive (iii) they are widely prevalent (iv) most of them can be easily mass cultured, and, (v) they can be integrated into organized pest management systems (Lancar and Krake, 2002). Many species of fungi have been reported for the control of water hyacinth, such as *Acremonium zonatum* (Sawatun) Gams. (Martyn and Freeman, 1978) *Alternaria eichhorniae* (Shabana et al. 1995) *Cercospora rodmanii* (Conway and Freeman, 1977; Charudattan, 1986), *Rhizoctonia solani* (Shahjahan et al., 1987) and *Myrothecium roridum* (Liyana and Gunasekera, 1989). In these fungal genera, several species of *Myrothecium* have been developed as a mycoherbicide for controlling the water hyacinth and others weeds such as *M. roridum* (Liyana and Gunasekera, 1989), *Myrothecium verrucaria* (Hoagland et al., 2007; Okunowo et al., 2010). There are numerous enzymes that *M. roridum* can produce such as the cellulases enzyme, which hydrolyzes the  $\beta$ -1,4-D-glycosidic bonds within the cellulose molecules of the plant (Akiba et al., 1995; Baer and Gudmestad, 1995; Zaldivar et al., 2001; Moreira et al., 2005; Okunowo et al., 2010). Mycotoxins are the series of macrocyclic trichothecenes such as epirodirin E, epiisoridrin E, roridin E, roridin H, trichoverrin A, trichoverrin B, verrucaridin A and verrucaridin J (Jarvis et al., 1985; Abbas et al., 2002).

Molecular data have been employed to identify and study the diversity of this fungus. Molecular-based identification techniques, including internal transcribed spacer (ITS) rDNA regions are effective tools for species identification. Due to the fact that ITS sequences are variable regions and rich in informative sites; hence, their usefulness for relationship of genus and species of fungi. Okunowo et al. (2013) successfully used ITS regions for identification of *M. roridum* isolated from water hyacinth in Nigeria. Moreover, several types of molecular marker such as amplified fragment length polymorphism (AFLP) is successful for study of the variability within and among fungal populations (Majer et al. 1996; Sibounnavong et al. 2012). The AFLP fingerprints are highly reproducible and used by unknown samples circumventing cumbersome multi-locus sequencing. Sibounnavong et al. (2012)

reported that AFLP markers could divided *Fusarium oxysporum* f. sp. *lycopersici* isolated from tomatoes in Thailand into 3 subgroups as low, moderate and high virulence.

The objectives of this study were to determine the tendency of using *M. roridum* to control the water hyacinth in Thailand. Moreover, the molecular based methods were performed to identify the fungal species and to evaluate the genetic diversity.

## MATERIALS AND METHODS

### Fungal isolation and preliminary identification

Water hyacinth leaf blight disease was observed, then 32 samples were collected from different localities of provinces in the central region such as Nakhon Pathom (6 samples), Pathum Thani (5 samples), Phra Nakhon Si Ayutthaya (2 samples), Suphan Buri (2 samples), Sumut Songkhram (2 samples), Samut Sakhon (3 samples), Ratchaburi (5 samples) and in the western region such as, Phetchaburi (4 samples) and Kanchanaburi (3 samples). Fungal pathogen was isolated from the leaves using the tissue transplanting method. The diseased leaf samples were cut into 0.5x0.5 cm samples and then the surface was disinfected with a 10% sodium hypochlorite solution for 5 min and then washed with sterilized distilled water before being moved to a potato dextrose agar (PDA). The cultures were incubated at 25°C under diurnal light. Single spore isolation was carried out for each of the pure cultures and maintained on PDA slants and deposited at the Fungal Collection, Department of Plant Pathology, Faculty of Agriculture at Kamphaengsaen, Kasetsart University, Kamphaeng Sean Campus (KKFC), Thailand. Each fungal isolate was identified on the basis of morphological characters such as colony color, color and shape of conidia and conidiophores (Barnett and Hunter, 1987).

### Pathogenicity test for controlling the water hyacinth

The most aggressive strains were primarily screened by colonial growth rate and spore number reproduction. Each isolate was grown on PDA and incubated at 28°C under diurnal light. The colonial dimension of *M. roridum* was measured every day for 9 days. The spore numbers were counted using a hemacytometer and the data were calculated for statistical analysis. The differences of mean value using ANOVA, was followed by Duncan's multiple range test (DMRT).

The most effective isolates were deposited on the water hyacinth leaves using the spraying method, with a spore suspension concentration of  $10^8$  spores/mL. The water hyacinths were planted until they reached the third generation, the size of leaves was 25-100 cm<sup>2</sup>. The experiment was conducted using a completely randomized design (CRD) with 10 replications in each treatment. The experiment group (treatment), consisted of 5 treatments and control treatment (N = 10 water hyacinth plants, each).

The healthy plant was inoculated with KKFC 390, KKFC 400, KKFC 402, KKFC 403 and KKFC 408 using spraying method with 10 mL of spore suspension. While, control treatment was performed by spraying with 10 mL distilled water. The plants were then placed in a growth chamber with 100% relative humidity for 24 h and then moved to their natural conditions. The disease severity was observed at seven days after inoculation using rating scale as follow as; 0 = no disease, 1 = 1-25% of leaf blight, 2 = 26-50% of leaf blight, 3 = 51-75% of leaf blight, 4 = 76-100% of leaf blight (Sultana and Ghaffar, 2009). The disease severities were analyzed for the mean value and standard deviation. The differences of mean value was shown using ANOVA,

followed by DMRT. Statistical analysis was performed using SPSS statistics software (version 15.0, Window). A  $P < 0.05$  was considered to be statistically significant. Moreover, the fresh weight of each water hyacinth was recorded before and after spraying, then the data were analyzed for the mean value and standard deviation.

### DNA extraction

All fungal isolates were cultured in potato dextrose broth (PDB) in a shaker for two to three days; the mycelia were then harvested on filter paper (Whatman No.1). Fungal mycelia were freeze-dried for 6-8 h using Lyophilizer, then stored at  $-20^{\circ}\text{C}$  for later use. Freeze-dried mycelium was ground in a mortar using liquid nitrogen, then 50 mg of the ground mycelium was suspended in 500  $\mu\text{L}$  of extraction buffer (200 mM Tris-HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA and 0.5% SDS) and incubated at  $65^{\circ}\text{C}$  for 30 min. 500  $\mu\text{L}$  of phenol: chloroform : isoamyl alcohol (25:24:1) was then added. After centrifugation at 13,000 rpm for 10 min, the upper aqueous phase was deproteinized by the addition of 1 volume of chloroform : isoamyl alcohol (24:1) and again centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was transferred to a 1.5 mL microtube containing 2  $\mu\text{L}$  of 10 mg/mL RNAase and incubated at  $37^{\circ}\text{C}$  for 30 min. After that 500  $\mu\text{L}$  of chloroform : isoamyl alcohol (24:1) was added and placed in a centrifuge at 13,000 rpm for 10 min, two volumes of absolute ethanol were added to the upper aqueous phase and the solution was stored at  $-20^{\circ}\text{C}$  for 1 h. The solution was again centrifuged at 13,000 rpm for 10 min. The precipitated DNA was washed with 200  $\mu\text{L}$  of 70% ethanol and was centrifuged at 13,000 rpm by 2 times, each having duration of 10 min. After drying, the DNA was dissolved with TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) by a modified method of Zimand et al. (1994).

### PCR amplification of ITS rDNA region

DNA samples of *M. roridum* were amplified in the ITS regions of rDNA using PCR amplification. Amplification of the ITS regions was done using 40  $\mu\text{L}$  PCR reaction each containing 5  $\mu\text{L}$  genomic DNA (50 ng), PCR buffer (1x), dNTP (0.2 mM), 5 pmole of each primer,  $\text{MgCl}_2$  (2.5 mM) and *Taq* polymerase (1 unit). The PCR amplification of the ITS regions was amplified using primer: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCTCTCCGTTATTGATATGC-3') (White et al., 1990). Amplification of the ITS regions was carried out using T professional Standard Gradient (Biometra) under the following condition:  $95^{\circ}\text{C}$  for 30 min, 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min, and finally at  $72^{\circ}\text{C}$  for 10 min. After amplification, 5  $\mu\text{L}$  of the PCR product was detected on 1% (W/V) agarose gels and then added to 0.1  $\mu\text{L}/\text{ml}$  GelStar (Nucleic acid Gel stain, 1,000 x concentrate in DMSO) in a TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.0). The PCR products were purified using Illustra™ MicroSpin S-400 HR columns (GE Health care UK Limited). The purified PCR products were sequenced at 1<sup>st</sup> BASE DNA Sequencing Services, Malaysia.

### Multiple sequences alignment and data analyses

Sequences generated from the ITS region of rDNA were aligned with other sequences retrieved from the GenBank database including *M. roridum* (JF724157 and EU927366), *Myrothecium gramineum* (FJ235084), *M. verrucaria* (AB778924) and *Fusarium oxysporum* (KC292853) using MEGA 5 (Tamura et al., 2011). Phylogenetic tree were calculated from the dataset using neighbour-joining (NJ) (Saitou and Nei, 1987) method in the same program. Node support was evaluated by bootstrap analysis

(Felsenstein, 1985) using 1,000 replications in the NJ analysis.

### DNA fingerprinting analysis using AFLP marker

The AFLP reactions were used as described by Vos and Kuiper, (1997) with the following modifications: Genomic DNA (500 ng) was digested with two combinations of restriction endonuclease *EcoRI* (50 Units) and *MseI* (10 units) in a mix of 10x digestive buffer, 0.5 M NaCl and BSA. The digested DNA fragments were ligated with their respective adapter pair (5 pmol *EcoRI* adapter and 50 pmol *MseI* adapter) of both enzymes in a reaction of T4 DNA ligase (1 unit), T4 DNA ligase buffer (1x) and 1 mM ATP. After the incubation at  $37^{\circ}\text{C}$  for 3 h, the restriction-ligation products were diluted 10 fold with distilled water.

The pre-selective amplifications were carried out with 1 selective nucleotide at 3' end of each primer in volume of 25  $\mu\text{L}$  of PCR buffer, containing PCR buffer (1x), dNTP (0.2 mM), each primer; *EcoRI* primer (5'-GACTGCGTACCAATTC-3') with additional one base (E+A, E+C and E+G), *MseI* primer (5'-GATGAGTCTGTAGTA-3') and additional one base (M+C, M+G, M+A and M+G) 5 pmole,  $\text{MgCl}_2$  (2.5 mM), *Taq* polymerase (0.5 unit). The pre-selective amplification was carried out using a thermal cycler for 20 cycles of 30 s duration at  $94^{\circ}\text{C}$ ; 60 s at  $56^{\circ}\text{C}$ ; 60 s at  $72^{\circ}\text{C}$  and held at  $16^{\circ}\text{C}$  for 15 min.

The selective amplifications were performed using selected combinations of primers with two or three selective nucleotides (Bleas et al., 1998) as shown in Table 1. All combination primers were screened to investigate the most suitable primers. These were carried out in 20  $\mu\text{L}$  of PCR buffer containing 5  $\mu\text{L}$  diluted (1:10) product of pre-selective amplified DNA, PCR buffer (1x), dNTP (0.2 mM), 5 pmole of each primer,  $\text{MgCl}_2$  (2.5 mM) and *Taq* polymerase (1 unit). The PCR amplifications were performed with an initial denaturation at  $94^{\circ}\text{C}$  for 30 s followed by 12 cycles at  $94^{\circ}\text{C}$  for 30 s, annealing at  $65^{\circ}\text{C}$ ; this was then reduced by  $1^{\circ}\text{C}$  for 30 s followed by an extension step at  $72^{\circ}\text{C}$  for 60 s. For each of the following 10 cycles, the annealing temperature was reduced by a further  $1^{\circ}\text{C}$ . The next 30 PCR cycles continued at  $94^{\circ}\text{C}$  for 30 s,  $56^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 60 s.

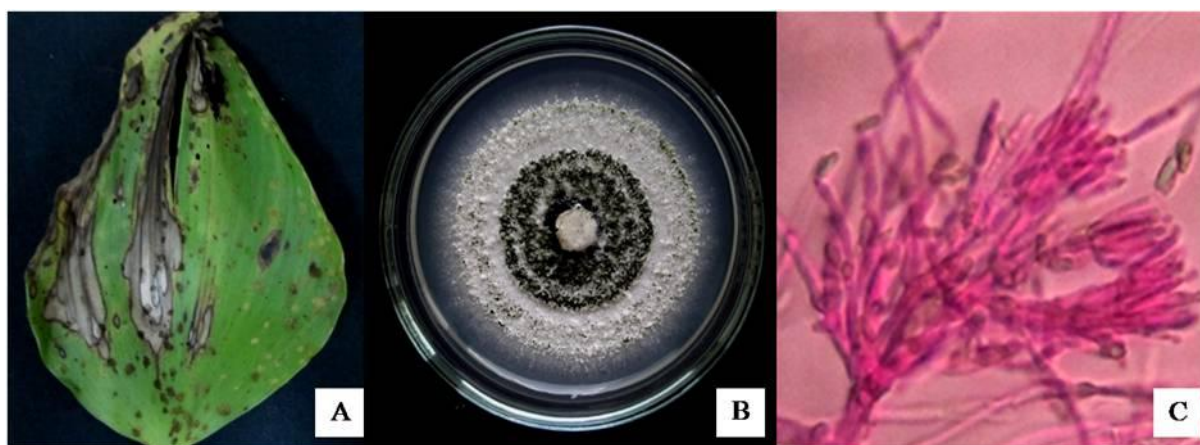
For gel analysis, the PCR products were mixed with 10  $\mu\text{L}$  of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.3% bromophenol blue and 0.3% xylene cyanol), then heated at  $95^{\circ}\text{C}$  for 3 min and quickly cooled on ice. Each sample (2  $\mu\text{L}$ ) was examined on a 5% polyacrylamide gel plus 7 M urea on a Model S2 sequencing gel electrophoresis apparatus. Electrophoresis was performed at a constant power of 50 W for 2.5 h. After electrophoresis, the gel plate was removed, fixed in 10% acetic acid for 30 min, and washed three times in distilled water for 2 min each time. The gel plate was then stained for 30 min in a silver solution (1 g of silver nitrate and 1.5 mL of 37% formaldehyde per liter) and then rinsed with distilled water. After staining, the gels were developed in a developer solution (30 g of sodium carbonate, 1.5 ml of 37% formaldehyde and 0.01 g of sodium thiosulfate) until the bands appeared. The staining was stopped by adding 10% acetic acid (fixing solution) for 1-2 min, rinsed with distilled water for 2 min and dried under a fume hood.

### Data analysis

Polymorphic bands were scored as binary data by 1 (present) or 0 (absent). The binary data were analyzed with the computer program NTSYS pc version 2.02 (Rohlf, 1993). An unweighted pair group method with arithmetic mean (UPGMA) cluster analysis was performed using the Jaccard's similarity coefficient. A dendrogram was generated with the tree option (TREE) and a cophenetic value was calculated with the COPH program in NTSYSpc. Bootstrap values were calculated with 1000 replications by the Winboot program (Yap and Nelson, 1996).

**Table 1.** Primer combinations used for screening in selective amplification.

<i>EcoRI</i>	<i>MseI</i>
5' –GACTGCGTACCAATT <u>C</u> C- 3'	5'-GATGAGTCCTGTAGT <u>A</u> C- 3'
5' –GACTGCGTACCAATT <u>C</u> A- 3'	5'-GATGAGTCCTGTAGT <u>A</u> G- 3'
5' –GACTGCGTACCAATT <u>C</u> G- 3'	5'-GATGAGTCCTGTAGT <u>A</u> AC- 3'
5' –GACTGCGTACCAATT <u>C</u> GT- 3'	5'-GATGAGTCCTGTAGT <u>A</u> AA- 3'
5' –GACTGCGTACCAATT <u>C</u> GC- 3'	5'- GATGAGTCCTGTAGT <u>A</u> ACG- 3'
5' –GACTGCGTACCAATT <u>C</u> AGC- 3'	5'-GATGAGTCCTGTAGT <u>A</u> CTA-3'
5' –GACTGCGTACCAATT <u>C</u> AGG- 3'	5'-GATGAGTCCTGTAGT <u>A</u> AC-3'
5' –GACTGCGTACCAATT <u>C</u> ATC- 3'	5'-GATGAGTCCTGTAGT <u>A</u> CGC- 3'
5' –GACTGCGTACCAATT <u>C</u> ACT- 3'	
5' –GACTGCGTACCAATT <u>C</u> CGC- 3'	



**Figure 1.** Blight symptoms causing by *Myrothecium roridum* on water hyacinth leaf with fungal mass (A) colony of *M. roridum* on PDA (B) and conidia and conidiophore of *M. roridum* under microscope (400 X) (C).

## RESULTS

### Fungal isolation and preliminary identification

The disease symptoms were teardrop-shaped leaf spot, rounded on the side facing the petiole and tapering to a narrow point in the direction of the leaf margin. Subsequently, the leaf spots turned to necrotic with dark brown margins with orange-white conidial masses (Figure 1A). Seventy isolates of *Myrothecium* sp. were identified using colony, conidia and conidiophore characteristics. Culture characteristics produced hyaline sporodochia and white margins. Spore masses were initially green, later a wet and shiny black. Sporulation often in concentric zones diffused in sporodochia (Figure 1B). The conidiophores were repeatedly branched and bearing phialides (Figure 1C). Conidia were rod shaped with rounded ends and colorless to pale olive green to black in mass. The conidial size was 3.36-4.88 x 0.73-2.10  $\mu$ m. All characteristics were consistent with description of *M. roridum* (Tulloch, 1972). Moreover, the colonial growth

observations indicated that there were five isolates including KKFC 390, KKFC 400, KKFC 402, KKFC 403 and KKFC 408; KKFC 408 had the highest colonial growth rate and spore production. Therefore, these isolates were selected for pathogenicity testing on the water hyacinth in greenhouse condition.

### Pathogenicity test for controlling the water hyacinth

Five isolates of *M. roridum* were sprayed on water hyacinth leaf and incubated in greenhouse condition. The results showed that there were variations among these isolates in disease virulence. In the preliminary stage of symptom, water hyacinth leaf was necrosis and developed into the form of a spot. After that the symptom became leaf blight with orange-white conidial masses on the water hyacinth leaf (Figure 2C). The average disease severity of *M. roridum* isolates KKFC 390, KKFC 400, KKFC 402, KKFC 403 and KKFC 408 were 1.8, 1.0, 1.8, 1.5 and 3.5, respectively, as shown in Table 2. There





**Figure 2.** Disease symptoms on water hyacinth recorded at 7 days after inoculation. Leaf blight symptoms on inoculated plant (A) control treatment (B) leaf blight symptoms with conidial mass (C).

**Table 2.** The disease severity on water hyacinth infected by *Myrothecium roridum* isolates.

<i>Myrothecium roridum</i>	Disease severity*
KKFC 390	1.8±0.4 <sup>b</sup>
KKFC 400	1.0±0.0 <sup>c</sup>
KKFC 402	1.8±0.8 <sup>b</sup>
KKFC 403	1.5±0.8 <sup>bc</sup>
KKFC 408	3.5±0.7 <sup>a</sup>
Control	0.0±0.0 <sup>d</sup>

\*Means followed by a common letter were significantly different by DMRT (P<0.05).

were statistical differences on disease severity among five isolates. The isolate KKFC 408 showed the highest disease severity on water hyacinth (P<0.05). The results were correlated to the fresh weight observed at 7 days after inoculation (Figure 3). The fresh weight of water hyacinth was reduced when compared with the control treatment. The results indicate that *M. roridum* isolate KKFC 408 had a potential for reducing the amount of water hyacinth in the water resource.

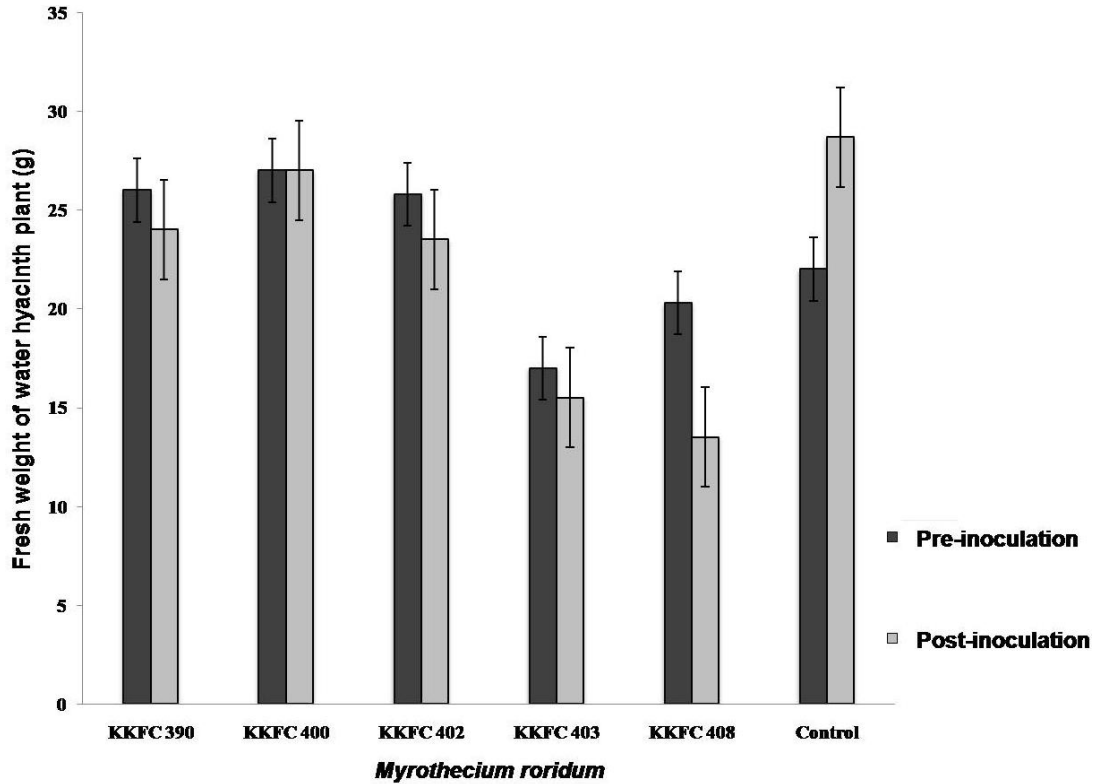
#### Molecular identification based on ITS region

Sequencing data of *M. roridum* isolates KKFC 390 (AB823651), KKFC 400 (AB823652), KKFC 402 (AB823653), KKFC 403 (AB823654) and KKFC 408 (AB823655) were 580-588 bp in length when aligned together with other sequences from the database. The similarity coefficient among five sequences of *M. roridum* was 99.99% when compared with the sequence of *M. roridum* obtained from the database (Figure 4). Moreover, the NJ clustering showed that five sequences of *M.*

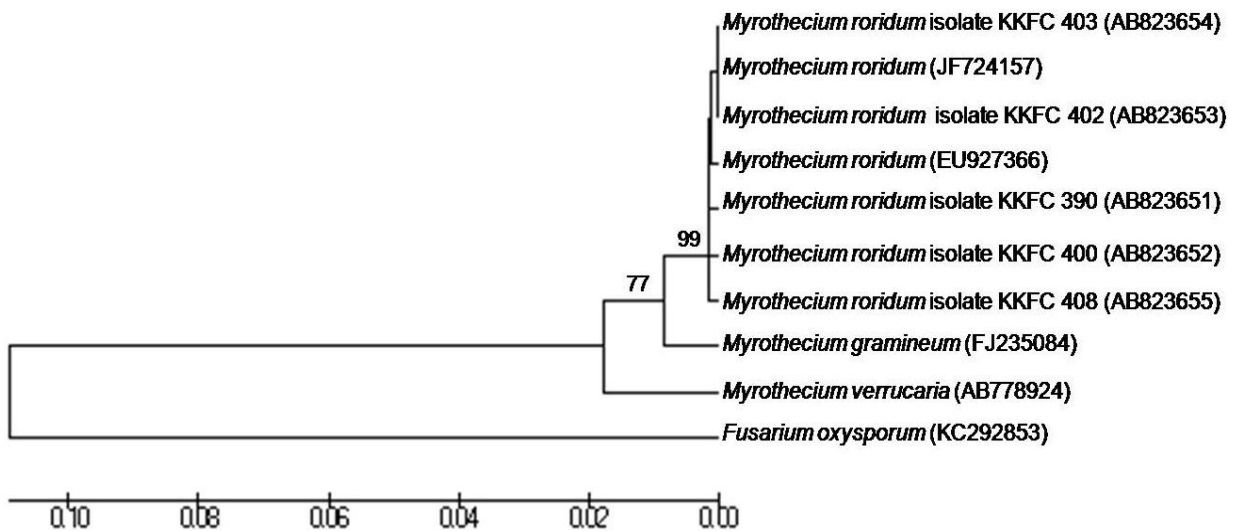
*roridum* were in same group with other sequences of *M. roridum* recorded in the database, this was supported by 99% bootstrap value and separated from other species such as *M. gramineum*, *M. verrucaria* and *F. oxysporum* (Figure 5). The result indicated that the five isolates could be identified as *M. roridum* based on ITS sequence which correlated to the morphological based identification.

#### DNA fingerprint analysis using AFLP markers

Twelve primer combinations were screened with five isolates of *M. roridum* for investigating a suitable primer combinations to be used in this study. The generated fingerprints were evaluated for overall clearness of the banding pattern and the number of polymorphic bands was recorded. The result showed that three of twelve primer recombinations such as *EcoRI*+*AGC*/*MseI*+*C* (19/51), *EcoRI*+*C*/*MseI*+*ACG* (24/ 57) and *EcoRI*+*A*/*MseI*+*CTA* (21/ 26) gave a high number of polymorphic bands. Therefore, these three primer combinations were used to observe the genetic variation among *M. roridum* isolates. The results showed that a total of 166 bands were generated with 116 polymorphic bands among the fifteen isolates of *M. roridum* and one isolate of *Fusarium* sp. (Figure 5). All isolates of *M. roridum* were separated from out group (*Fusarium* sp.) with support by 100% bootstrap value and cophenetic values (r) = 0.97. Cluster analysis divided all isolates of *M. roridum* into 4 subgroups at Jaccard's coefficients similarity ≥0.5. The subgroup 1 consisted of isolates KKFC384, KKFC390, KKFC401, KKFC388 and KKFC398; the subgroup 2 included isolates KKFC407, KKFC409 and KKFC410; the subgroup 3 included isolate KKFC403; subgroup 4 consisted of isolates KKFC385, KKFC387, KKFC411, KKFC406 and KKFC389 (Figure 6).



**Figure 3.** The fresh weight of water hyacinth plants measured before inoculation (black) and 7 days after spraying inoculation (gray).



**Figure 4.** Neighbor-joining phenogram showing the phylogenetic relationship within the species of *M. roridum* and among species of *Myrothecium* based on ITS rDNA sequences. Bootstrap values are indicated on the branches (1000 replication).

**DISCUSSION**

*Myrothecium roridum* was specific pathogen to water

hyacinth and could cause leaf blight disease. In addition, pathogen produced enzymes and phytotoxic metabolites. Dagno et al. (2012) report showed that *M. roridum* could



**Figure 5.** AFLP band patterns of *Myrothecium roridum* isolates obtained from three primer recombinations including *EcoRI*+*AGC*/*MseI*+*C*, *EcoRI*+*C*/*MseI*+*ACG* and *EcoRI*+*A*/*MseI*+*CTA*. Lane M = 100 bp Plus DNA Leader; the polymorphic bands are indicated with arrows.

be used for controlling water hyacinth population growth because *M. roridum* produced and released several metabolites such as enzymes, antibiotics and mycotoxins.

There are numerous enzymes that *M. roridum* can produce and release onto the water hyacinth leaf such as cellulases enzymes which hydrolyze the  $\beta$ -1,4-D-glycosidic bonds within the cellulose molecules of plant (Akiba et al., 1995; Baer and Gudmestad, 1995; Zaldivar et al., 2001; Moreira et al., 2005; Okunowo et al., 2010), the series of macrocyclic trichothecenes such as epiroridin E, epi-isororidin E, roridin E, roridin H, trichoverrin A, trichoverrin B, verrucaridin A and verrucaridin J

(Jarvis et al., 1985; Abbas et al., 2002). Wang et al. (2007) confirmed that *M. roridum* produced mycotoxin in group trichothecenes namely Roridin and Isororidin. While, Okunowo et al. (2010) reported that *M. roridum* is capable of producing cellulase and xylanase in submerged cultures containing different carbon sources. In this study, seventy isolates of *M. roridum* were isolated from the water hyacinth leaf blight disease from different geographical area in Thailand.

Five of them were found to be more aggressiveness strains; these strains had pathogenicity when tested on the water hyacinth. The results indicated that *M. roridum* isolate KKFC 408 had the highest disease severity when compared with the other isolates and the control treatment. In these fungal genera, several species of *Myrothecium* have been developed as a mycoherbicide for controlling the water hyacinth and other water weeds such as *M. roridum* (Liyanage and Gunasekera, 1989) and *M. verrucaria* (Hoagland et al., 2007; Okunowo et al., 2010).

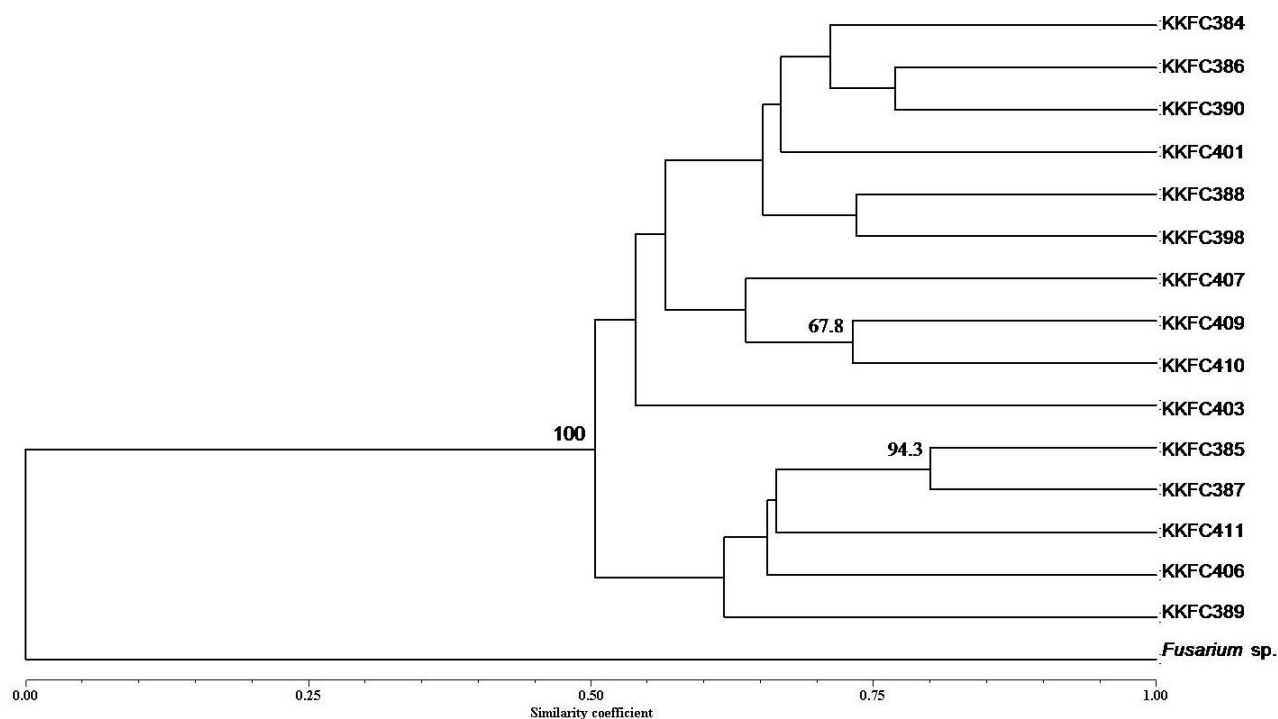
In the previous study, Jonniaux et al. (2004) reported on phylogeny obtained from ITS sequences data of *Myrothecium* species including *Myrothecium leucotrichum*, *Myrothecium cinctum*, *M. roridum*, *Myrothecium verrucaria*, *Myrothecium atroviride*, *Myrothecium gramineu* and *Myrothecium inundatum*. The information indicated that the ITS region genes could be used for identification and differentiation among these species of *Myrothecium*. The present study agrees with Jonniaux et al. (2004) and Okunowo et al. (2013), five isolates of *Myrothecium* were consistent with the sequences of *M. roridum* obtained from the GenBank database and are separate from other species of *Myrothecium*.

According to the AFLP analysis, the results showed that the AFLP groups were not correlated to the geographical area. Within the group, there were the fungal isolates collected from different area. The results showed that there was movement of conidia among geographical areas or provinces.

This process is probably relative to genotype flow in which many genes move together as a block in asexual spores. Genotype flow occurs only for organisms that have asexual reproduction in their life cycle (McDonald, 2004). As an example, *F. oxysporum* has only asexual cycle, the genotype of *F. oxysporum* f.sp. *melonis* (wilt on melon) moves from North America to Israel on the muddy boots of an agricultural scientist. Thus, the entire set of alleles in the clone is introduced into a new population. If this clone has high degree of fitness, it can become established in the new location (McDonald, 2004).

In the previous report, Mohamed et al. (2003) used AFLP fingerprinting to study genetic relationships within and between natural populations of five *Fusarium* species, that is, *F. oxysporum*, *Fusarium solani*, *Fusarium moniliforme* and *Fusarium semitectum* isolated from Egypt. The results of the AFLP analysis enabled the classification of *Fusarium* isolates into f major clusters.





**Figure 6.** Dendrogram of *Myrothecium roridum* isolates based on the binary matrix of polymorphic bands, using the Jaccard's similarity coefficient and UPGMA algorithm. Bootstrap values above 50% from 1,000 replicates are indicated for the corresponding branch.

There was no correlation between AFLP data and geographic origin, host genotype.

## Conclusion

*Myrothecium* is an effective fungal pathogen to control the water hyacinth in Thailand. The molecular based identification confirmed the species of *Myrothecium* as *M. roridum*. The AFLP based evaluation indicated that the conidial movement occurs among the geographical area, and then they can establish and survive in the new locality.

## Conflict of interests

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

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Full Length Research Paper

# Bacterial pathogens associated with infectious diarrhea in King Khalid University Hospital, Riyadh Saudi Arabia 2005-2010

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Diarrhoeal diseases are major cause of morbidity worldwide. In developed and developing countries, *Salmonella*, *Shigella* and *Campylobacter* species are the main bacterial causes. This study aimed to investigate the occurrence and antimicrobial susceptibility of bacteria causing diarrhea in Riyadh, during a six years period. This study was performed in Riyadh capitol of Saudi Arabia between January 2005 to December 2010. The data was obtained from request forms for stool examination of patients complaining of diarrhea. Specimens were cultured; isolates were identified biochemically and serologically following conventional methods. Two percent of specimens tested were positive, 89.1% of these grew *salmonella* species, 5.6% *Shigella* species and 5.3% grew *campylobacter jejuni*. Most of these isolates were from the age group >1-5 years old. Cefotaxime was the most active agent against *salmonella* and *shigella*, while erythromycin was the most active agent against the *C. jejuni*. *Salmonella*, *Shigella* and *Campylobacter* species were the main isolates from stool of patient with diarrhoea. Cefotaxime was the most active agent on *Salmonella* and *shigella* species, while erythromycin was the most active agent against *C. jejuni*.

**Key words:** Diarrhea, *Salmonella*, *Shigella campylobacter*, Saudi Arabia.

## INTRODUCTION

Diarrhoeal diseases are one of the five leading causes of death worldwide (Wanke et al., 2013a). Globally, one in ten children deaths results from diarrhoeal diseases during the first 5 years of life which results in 800,000 fatalities worldwide annually; most of these occurring in subsharan countries (Kotloff et al., 2013). In developed countries (USA) acute infectious diarrhea results in more

than 1.5 million patient visits to doctors and 200,000 hospitalization annually (Kotloff et al., 2013). In developed countries viruses predominate as main causes of diarrhea but the main bacterial causes of diarrhea include *Campylobacter*, *Salmonella*, *Shigella* species, *Aeromonas* species and *Escherichia coli* (Wanke et al., 2013b). However sporadic cases of diarrhea due to vibrio

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**Table 1.** Shows the total number of specimens tested, number and percentage of negative and positives specimen for pathogens in each of the six years of the study.

Parameter	2005	2006	2007	2008	2009	2010	Total
Positive (%)	114 (3)	98 (3)	99 (2.9)	104 (2.5)	62 (1.4)	74 (1.8)	551 (2.4)
Negative	3480	3399	3262	4041	4292	4390	22,864
Total	3587	3303	3365	4142	4378	4140	22,915

cholerae and Yersina do occur in these countries. The types of etiological bacterial agents of diarrhea in developing countries are similar to those in developed countries; however *Shigella*, *Salmonella* and vibrios predominate more in developing countries (Kumar and Subitha, 2012).

The aim of this study was to investigate the role of bacterial pathogens in cases of diarrhea in a major teaching hospital Riyadh the capital of the Kingdom of Saudi Arabia during a six year period. The study also aimed to see the antimicrobial resistance of these bacterial pathogens and compare it to previous studies in the area of the study, the kingdom of Saudi Arabia as a whole and some worldwide studies.

King Khalid University Hospital (KKUH) is the main teaching hospital in the Kingdom of Saudi Arabia with about 900 bed capacity. It is a tertiary care hospital serving a population of 2-3 millions of local and expatriates nationals. To the best of our knowledge, only few such studies were done in recent years from this area. The rapid worldwide development of bacterial resistance to antimicrobial agents necessitates such studies to follow up these changes.

## MATERIALS AND METHODS

Laboratory request forms for stool specimens' examination were used to retrospectively get data related to patients from whom these specimens were collected during the period of January 2005 to December of 2010. Data collected included demographic data of patients, documented clinical features, identification and antimicrobial susceptibility of bacterial pathogens isolated.

### Laboratory testing

Stool specimens were processed by culturing directly onto MacConkey agar, XLD agar plates and salenite F enrichment broth. The latter medium was sub-cultured onto MacConkey and Hektoen agars after overnight incubation at 35°C.

All plates were examined for *Salmonella* species, *Shigella* species and *E. coli* by standard microbiological methods. The MicroScan automatic system (Dade Behring a Siemens Company) was used for biochemical identification. Commercial anti-sera were used for fine focused serological identification of the isolates (Difco laboratories detroit, Mich USA).

For detection of *Campylobacter jejuni* and *Campylobacter* species, stools were cultured on Campy plate selective medium incubated at 42°C for 48 h under microaerophilic conditions. Identification of *C. jejuni* was done by the conventional methods

including gram stain, oxidase test, hippurate utilization and nalidixic acid susceptibility. The *Campylobacter* isolates were identified as *C. jejuni* or otherwise as *Campylobacter* species.

Laboratory processing of faecal specimens for *Vibrio* species and *E. coli* (0157 H7) as well as other rarely occurring pathogens was done if requested for or indicated by clinical features. This was done by inoculating faecal specimens on Thiosulfate-citrate bile salts (TCBS) for vibrios and sorbitol MacConkey for *E. coli* (0157 H7) respectively.

Detection of *Yersinia enterocolitica* is done by cold temperature enrichment procedure by incubating phosphate buffered saline incubated at 4°C for three weeks.

For detection of *Aeromonas* species group of organisms, stool samples were plated on sheep blood with incorporation of ampicillin (30 µg/ml) agar incubated at 35°C for 18 to 24 h. Presumptive haemolytic *Aeromonas* colonies if found were picked and tested for oxidase production. All oxidase positive colonies were further identified by the MicroScan automated system.

## RESULTS

From January 2005 to December 2010 a total of 22,311 stool specimens were submitted for culture, 551 specimens were positive for some enteric bacterial pathogens, indicating 2% positivity. Of these *salmonella* species were the commonest bacterial pathogens isolated with a number of 491 shown (89.1%) of the total positive isolates followed by *C. jejuni* with a number of 29 and a percentage of 5.3% then *Shigella* species accounting for 31 isolates and 5.6% of the total positives. No *Aeromonas* species, *Yersinia enterocolitica* vibrios or *E. coli* 0157 H7 were isolated in this study.

Table 1 shows the numbers and percentages of the total negative and positive specimens from the total specimens tested. The ranges of the positive specimen numbers ranges between 114 in the year 2005 and 62 in the year 2009. However the percentage of positivity of specimen each year does not differ much in different years, it range between (3 and 1.4%) in the years 2005 and 2009, respectively.

Table 2 shows the number of patients in different age groups from whom the enteric bacterial pathogens were isolated. The age group >1-5 years was the most affected age range with a number of 194 patients (35.2%) having positive results, followed by the >15-50 age group represented by 187 patients (33.9%) with positive results. The age group <1 year of age was the least affected age group showing 3 patients (0.5%) with positive results.

Table 3 shows the number and percentages of

**Table 2.** Number and percentage of patients in different age groups from whom the enteric bacterial pathogens were isolated during the six year period of the study (No. 551).

Age (years)	Number	Percentage
<1	3	0.5
1-5	194	35.2
>5-15	116	21.1
>15-50	187	33.9
>50	51	9.3
Total	551	100

**Table 3.** Shows the numbers and percentage of patients nationality from whom the enteric pathogen were isolates No. (551).

Nationality	Frequency	Percentage
Saudi	427	78.3
Indian	24	4.4
Filipino	20	3.7
Pakistan	14	2.6
Egyptian	13	2.4
Sudanese	9	1.7
Jordania	8	1.5
Indonesia	7	1.3
Yemeni	7	1.3
Unknown	6	1.1
Syrian	5	0.9
Banglade	4	0.7
Algerian	1	0.2
Eriteria	1	0.2
Iraqi	1	0.2
Lebanese	1	0.2
Nepali	1	0.2
Srilanka	1	0.2
Tanzania	1	0.2
Total	551	100

nationality of patients from whom the enteric pathogens were isolated. The majority of the patients with positive stool culture were Saudi 427 (78.3%), followed by Indian 24 (4.4%) and Filipinos 20 (3.7%). Male gender was predominant as it is represented by 322 patients (58.4%) versus female showing 229 patients (41.6%). The different nationalities have different habits and different accommodation facilities.

Figure 1 shows the percentage of resistance of *C. jejuni* isolates to antimicrobial agents tested. The highest resistance was to cephadrine (96%) followed by nalidixic acid (79%). However erythromycin was the most active agent against these isolates.

Figure 2 shows the percentage of resistance of *shigella*

species isolates tested to the antimicrobial agents used. The highest activity against these isolates was shown by cefotaxime which shows 0% resistance, followed by ciprofloxacin showing 6.7% resistance while the highest resistance was shown to ampicillin (53.30%).

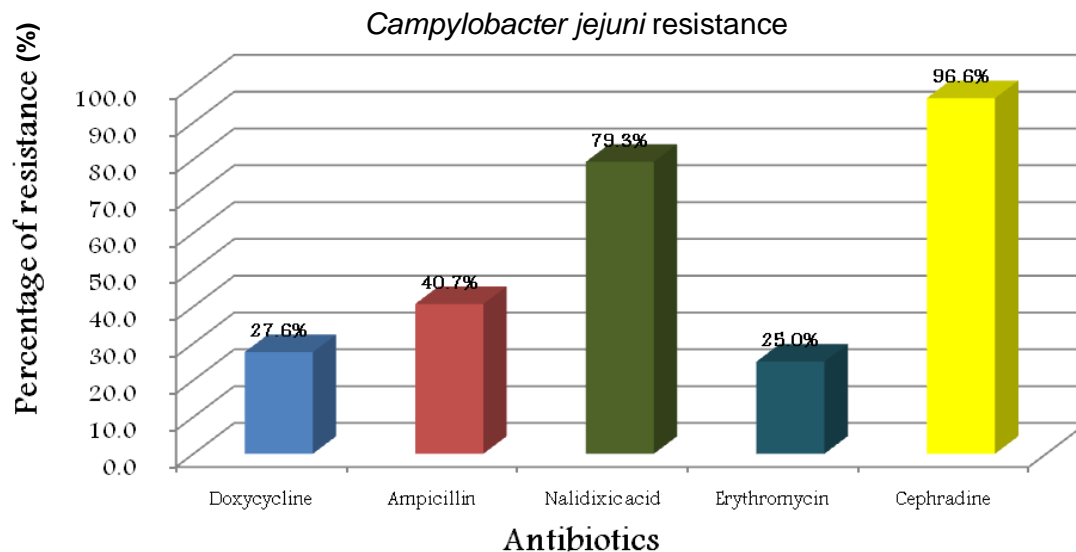
Figure 3 shows the percentage of resistance of *salmonella* species isolates to antimicrobial agents. The highest activity was shown by cefotaxime with 2.10% resistance followed by chloramphenicol with 8.20% resistance. However the highest resistance was shown to nalidixic acid (43.30%) and ciprofloxacin resistance to (34.30%) and then cephalothin with (33.30%) resistance.

## DISCUSSION

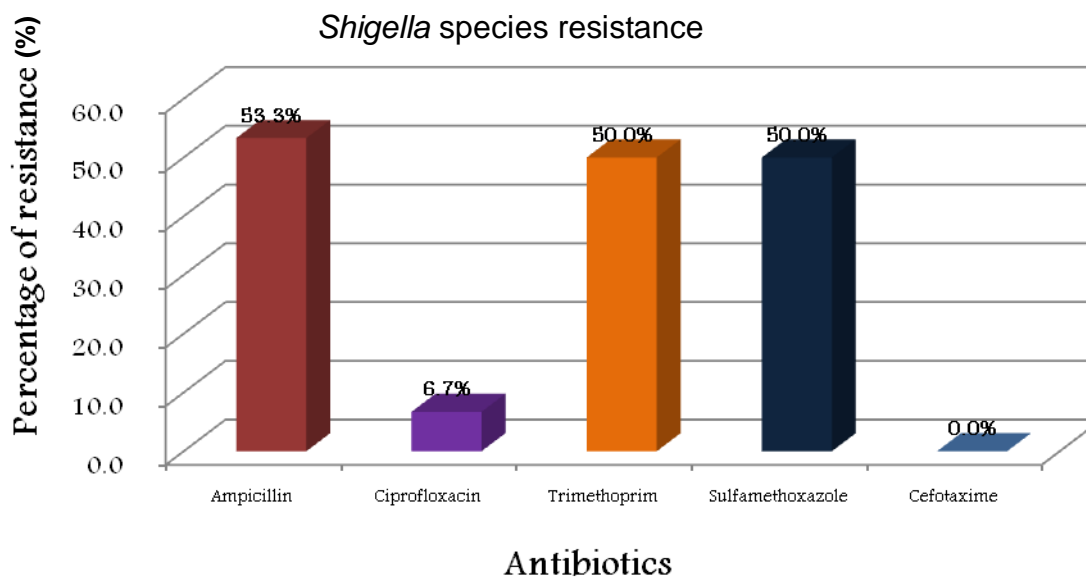
Diarrheal diseases have great impact on human health especially on children (Kosek et al., 2003). A study comparing this burden between the periods of 1954-1979 and the period of 1990-1989 showed a decline of mortality in children under 5 from an estimate of 13.6 to 5.6 to 4.9/1000 per year (Kosek et al., 2003). Diarrheal diseases are major causes of morbidity with attack rates ranging from 2 to 12 per person per year in developed and developing countries (Glass et al., 1996). The causes of diarrhoea include a wide range of virus (Glass et al., 1996; Guerrant et al., 1990) like rota virus (Tucker et al., 1998), bacteria (Mastson and Estes, 1990) and parasites (Piekering, 1990).

This retrospective study of ours was based on information from laboratory reports. Accordingly the information on clinical features of the patients may not be complete. However important facts derived from this study are the rate of isolation and antimicrobial susceptibility of bacterial gastrointestinal pathogens. It clearly reflects the importance of *Salmonella* species as cause of diarrhoea in this part of the world as documented by other studies (Kosek et al., 2003). The antimicrobial susceptibility of these isolates in our study showed high resistance to ampicillin a result that concurs with a previous studies from our institution (Somily et al., 2012) and others (Holmes et al., 1996) but differ from other (Avendaño et al., 1993; Hardy et al., 1994). This study of ours also shows the importance of *C. jejuni* as a cause of diarrhea in this region as has been reported in developed and developing countries (Guerrant et al., 1999). Our low results on *shigella* species isolates are similar to those from developed countries but differ from those reported from developing countries where *Shigella* species are not an uncommon cause of diarrhea (Koopman et al., 1984). These results rank the Kingdom of Saudi Arabia with, its well-developed water and drainage system at least in the area of our study, between the developing and developed countries.

During this period of the study no *Clostridium difficile*, *Yersinia*, *Vibrio*, *E. coli* 0157 H7, *Aeromonas* or *Pleisiomonas* species were isolated. These pathogens are either rare in our area or other parts of the Kingdom



**Figure 1.** Percentage of resistance of *Campylobacter jejuni* to commonly used antibiotics (No.78).



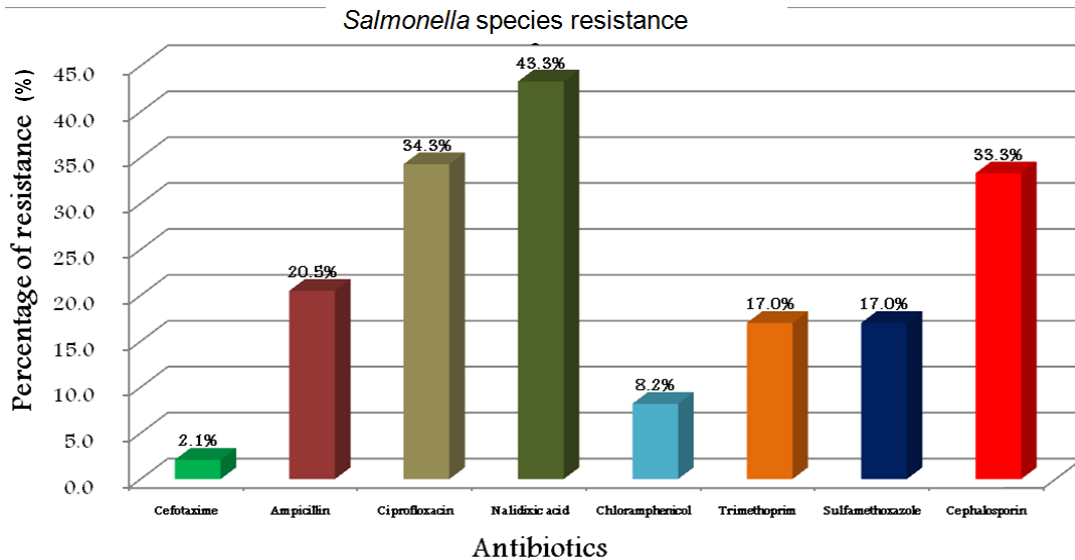
**Figure 2.** Percentage of resistance of *Shigella* species to commonly used antibiotics (No.46).

(CDC, 2003). They may be unexpected or not looked for in the kingdom as a whole, like other pathogens. However they may only be looked for during the Hajj season or places where people from different nationalities attend this occasion. However more investigation is needed in this aspect to look for these rarely isolated pathogens (Dennehy, 2005; Albert et al., 1999). An explanation for the reduction of the rate of the isolation of enteric pathogens in our study may be due to the high selectivity of our stools culture media. As recommended by CLSI we advise that the quality and

selectivity of these media should be checked using ATCC organisms to test the performance and selectivity of enteric media before use (CLSI, 2004).

*Shigella* species known as common cause of bacterial gastroenteritis after *Salmonella* in developed and developing countries (Koopman et al., 1984; CDC, 2003) was detected in only a small number of our cases (Mikhail, et al., 1990).

*C. jejuni* has been recognized as the most common cause of sporadic bacterial gastroenteritis in many countries (Sojo et al., 1982; Blaser et al., 1982). Again



**Figure 3.** Percentage of resistance of *Salmonella* species to commonly used antibiotics (No.427).

the fastidious nature of this organism and absence of quality control (QC) of *Campylobacter* media raises the possibility of under detection of these pathogens in our study (CLSI, 2010).

In our study around 80% of *C. jejuni* were resistant to Nalidixic acid which is a surrogate that indicates ciprofloxacin resistance and this coincides with recent literature of fluoroquinolone resistance. This may be due to the use of these agents in poultry and in veterinary medicine in general, which has increased the reservoir of resistant *Campylobacter* species (Smith et al., 1999) although ciprofloxacin was not tested on *Campylobacter* during this study we concluded this from resistance to Nalidixic acid.

Resistance of *C. jejuni* to erythromycin in this study was 70% which may result from mutations in the genes encoding ribosomal proteins or it may also result as has been claimed before to be associated with decreased permeability of the cell envelope as in enterobacteriaceae, including plasmid mediated mechanism (Burnens et al., 1996; Steinbigel, 1990).

Our results on *C. jejuni* resistance to macrolides is consistent with other studies as it has been previously noticed that the incidence of macrolide resistance among *C. jejuni* and *Campylobacter coli* is highly variable with respect to the origin country of isolation. It is lowest in Canada (12%) and Germany (0%) (*C. jejuni*) (Gibreel et al., 2005; Gaudreau and Gilbert, 2003; Lubber et al., 2003) and highest in Taiwan (*C. coli*) 50% (Li et al., 1998) and Nigeria 79.8% (Smith et al., 1999). However a study on macrolide resistance in *C. jejuni* in Sweden showed stable pattern (Engberg et al., 2001; Osterlund et al., 2003).

The high rate of resistance of macrolide in *campylobacter* species may be related to the extensive

veterinary use of macrolides (Moore et al., 1996; Ishihara et al., 2004) which is more prominent in *C. coli* than *C. jejuni* as in Japan all *C. jejuni* were susceptible to macrolide and 48.4% of *C. coli* were resistant (Ishihara et al., 2004). As there is study from our region in relation of antimicrobial in no veterinary medical. It is difficult to reach a conclusion concerning the effect of this as resistance of *campylobacter* species in our study.

## Conclusion

*Salmonella* species, *Shigella* species and *C. jejuni* are the most common enteric pathogens isolated from patients complaining of diarrhea in a teaching hospital in Riyadh Saudi Arabia. Pathogen like, *E. coli* 0157 H7 vibrios, *Aeromonas*, *Plesiomonas* were not encountered in this study.

Resistance of *Salmonella*, *Shigella* and *Campylobacter* concur with different results from developing countries. Media used for culture of stool should be quality controlled to make sure that some enteric pathogens are not missed or overlooked.

## Conflict of Interests

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

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## Full Length Research Paper

# Detection of selected anaerobic pathogens in primary and secondary endodontic infections in a Turkish population

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The aim of this investigation was to examine the presence of 8 bacterial anaerobic species in endodontic samples from patients with primary and secondary infection. The association of clinical signs and symptoms with constituent species were also evaluated. Microbial samples were obtained from 72 teeth with primary endodontic infection and 35 teeth with secondary endodontic infection. DNA was extracted from samples and analyzed with a polymerase chain reaction (PCR)-based identification assay. Medical and dental histories were obtained from each patient. The prevalence of the targeted bacterial species was recorded for each case and descriptive statistical analyses were performed using the Pearson Chi-squared test. Nucleid acid amplification method (NAAM) analysis showed that all specimens were positive at least for 1 or more samples in primary and secondary infection teeth. The most frequently detected bacteria in all specimens were *Porphyromonas gingivalis*, followed by *Porphyromonas micros*, *Porphyromonas endodontalis*, *Fusobacterium nucleatum*, *Porphyromonas intermedia* and *Tannerella forsythia*, respectively. The percentages of all selected bacteria found in primary infection group were higher than secondary infection group except for *Porphyromonas intermedia*. However, statistically significant difference was found only for *T. forsythia* and *F. nucleatum* which were higher percentage in primary infection than in secondary infection group. There was a significant association between tenderness to percussion and *P. gingivalis* ( $p < 0.05$ ), pain with *Porphyromonas melaninogenica* ( $p < 0.05$ ) and swelling with both *P. gingivalis* ( $p < 0.05$ ) and *P. melaninogenica* ( $p < 0.05$ ). Findings indicated that the prevalence of some species found in the primary infection group were higher than in the secondary infection group. In this study there was a significant association between tenderness to percussion and *P. gingivalis*, pain with *P. melaninogenica* and swelling with both *P. gingivalis* and *P. melaninogenica*.

**Key words:** Primary endodontic infection, secondary endodontic infection, anaerobic bacteria, nucleid acid amplification method (NAAM).

## INTRODUCTION

Apical periodontitis is caused by bacteria of infected root canals (Kakehashi et al., 1965). Necrotic root canals are typically polymicrobial, with nearly equal proportions of

Gram-positive and Gram-negative bacteria, and are dominated by anaerobic bacteria (Siqueira, 2002). In contrast, the microbial flora in secondary endodontic infec-

tions have been described as mono-infections or infections including a few Gram-positive bacterial species, with approximately equal proportions of facultative and obligate anaerobes (Sundqvist et al., 1998; Pirani et al., 2008).

Infections of the root canal system with facultative and obligate anaerobic bacteria have been associated with different clinical signs and symptoms (Jung et al., 2000; Gomes et al., 2004; Siqueira et al., 2004; Cavrini et al., 2008). Significant associations were found between individual clinical features and the following pairs of species: *Peptostreptococcus* spp., *Prevotella melaninogenica*, *P. micra* are associated with pain, *P. micra* and *Prevotella* spp. is associated with swelling and *Prevotella* spp., *Eubacterium* spp. and *Peptostreptococcus* spp. are associated with wet canals (Gomes et al., 1996). It is well known that most periodontal pathogens like *P. gingivalis* and *P. endodontalis* are also endodontic pathogens which are the key organisms in adult periodontitis and frequently found in root canal infections. (van Winkelhoff et al., 1985). *T. forsythia* is strongly associated with chronic periodontitis, an inflammatory disease of the tooth-supporting tissues, leading to tooth loss (Settem et al., 2012). *F. nucleatum* appears to be associated with the development of the most severe forms of inter appointment endodontic flare-ups (Chávez de Paz Villanueva, 2002). *P. melaninogenica* and *P. micra* is associated with pain and swelling (Drucker, 2000).

Recent findings revealed differences in the prevalence of several species between distant geographical locations (Baumgartner et al., 2004; Siqueira et al., 2005). Studies investigating the polymicrobial etiology of apical periodontitis indicated that the bacterial community profiles significantly vary between patients of different locations (Baumgartner et al., 2004; Siqueira et al., 2005). Until now, no previous investigation has been reported the presence of obligate anaerobic bacteria in endodontic samples from the root canal microbiota of patients from Cukurova region of Turkey.

The purpose of this investigation was to examine the presence of 8 bacterial anaerobic species (*Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, *Prevotella melaninogenica*, *Prevotella nigrescens*, *Tannerella forsythia*, *Parvimonas micra*), which are associated with endodontic infections, from patients with primary infection and secondary infection by employing Nucleic acid amplification methods (NAAM) and the association of clinical signs and symptoms with the constituent species.

## MATERIALS AND METHODS

One-hundred and seven patients, who were referred to Cukurova University Dental School for endodontic treatment or retreatment

were included in the study. Seventy-two teeth presented necrotic pulp and 35 teeth had previously been root-filled and showed clinic and radiographic evidence of apical periodontitis. All patient related procedures used in this study were approved by the Ethical Committee of the University of Cukurova and informed consent was obtained from each patient.

## Clinical signs and symptoms

Medical and dental histories were obtained from each patient. Age, gender, tooth type, pulp status, pain, history of previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus, presence of swelling, history of previous and present antibiotic therapy were recorded. Periodontal probing depths of selected teeth were also recorded and periodontal pockets more than 4 mm deep were excluded from the study because of possible endodontic-periodontal infection. For all teeth the presence of periapical radiolucency was assessed using the periapical index (PAI), determined with a paralleling X-ray technique (Orstavik et al., 1986). Patients who had not been treated with antibiotics in the preceding 3 months and who had no systemic diseases were included in the study. For necrotic teeth, an electric pulp test was conducted.

## Sampling procedures

For sampling, each tooth was cleaned with pumice and isolated with a rubber-dam. Gingival barrier was used between the teeth and the rubber-dam for each case. The tooth and surrounding field were cleaned with 35% hydrogen peroxide and decontaminated with a 5% sodium hypochlorite (NaOCl) solution. After disinfection, the coronal restorations were removed. Endodontic access was completed with a sterile high speed carbide bur. After completion of the endodontic access, the tooth, clamp and adjacent rubber-dam were once again disinfected with 5% NaOCl and then inactivated with sodium thiosulphate to avoid interference with the bacteriological sampling. A microbiologic sample was taken from the root canal after discrete filing motion with sterile #15 K-file and three sterile paper points. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Afterwards paper points were placed into the canal, with each left for 1 min for absorbing all the fluids present within them. These paper points were then transferred to cryo-tubes containing TE buffer and immediately frozen at -80°C.

In cases with secondary infections, pre-existing root canal fillings were removed using a Gates-Glidden drill and the apical material was retrieved using K-type files without the use of chemical solvents. Sterile saline solution was introduced into the canal to remove any remaining materials and to release the debris. The same procedure was used for the root canal sampling.

## Polymerase chain reaction (PCR) assays

DNA extraction of samples was performed using the Invitrogen PureLink Genomic DNA Mini Kit (Lot No: 449092, Carlsbad, CA 92008) according to the manufacturers recommendations. Reference DNA from the selected microorganisms: *Fusobacterium nucleatum*-ATCC 25586, *P. gingivalis*-ATCC 33277, *P. endodontalis*-ATCC 35406, *P. intermedia*-ATCC 15032, *P. melaninogenica*-ATCC 25845, *P. nigrescens*-ATCC 33563, *T. forsythia*-ATCC 43037 *P. micra*-ATCC 33270 was extracted. A negative control (without DNA) and a positive control (DNA of

**Table1.** PCR primers, expected amplicon sizes and thermocycling conditions for endodontic pathogens.

Target microorganism	Primer sequences (from 5'to 3')	Amplicon size (bp)	Thermocycling conditions	Cycle
<i>Fusobacterium nucleatum</i>	AGA GTT TGA TCC TGG CTC AG GTC ATC GTG CAC ACA GAA TTG CTG	360bp	94°C 1 min, 60°C 1 min, 72°C 2.5 min	30
<i>Porphyromonas gingivalis</i>	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404bp	94°C 30 s, 60°C 1 min, 72°C 2 min	36
<i>Porphyromonas endodontalis</i>	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATG TC	672bp	94°C 30 s, 60°C 1 min, 72 °C 2 min	36
<i>Prevotella intermedia</i>	TTT GTT GGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCT GCG T	575bp	95°C 30 s, 55°C 1 min, 72°C 2 min	36
<i>Prevotella melaninogenica</i>	CGT CAT GAA GGA GAT TGG ATA GAA CCG TCA ACG CTC	122bp	95°C 15 s, 54°C 30 s, 72°C 1 min	25
<i>Prevotella nigrescens</i>	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804bp	94°C 30 s, 55°C 1 min, 72°C 1 min	36
<i>Tannerella forsythia</i>	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641bp	94°C 30 s,55°C 30 s, 72°C 1 min	35
<i>Parvimonas micra</i>	AGA GTT TGA TCC TGG CTG AG ATA TCA TGC GAT TCT GTG GTC TC	207bp	94°C 1 min, 60°C 1 min, 72°C 2.5 min	30
Universal rDNA	26S GAT TAG ATA CCC TGG TAG TCC AC CCC GGG AAC GTA TTC ACC G	602bp	95°C 30 s, 60°C 1 min, 72°C 1 min	36

*Fusobacterium nucleatum*, *P. gingivalis*, *P. endodontalis*, *P. intermedia*, *P. melaninogenica*, *P. nigrescens*, *T. forsythia*, *P. micra*) were used. The DNA concentrations of reference strains and clinical endodontic samples were assessed by spectrophotometer at the 260 nm absorbance level.

Table 1 lists the PCR primers, predicted amplicon lengths and thermocycling conditions for the bacterial species tested. Initially, a universal eubacterial primer pair was used to detect DNAs from all bacterial species present in the sample (Dahlen et al., 2000; Ashimoto et al., 1996). The primers were purchased from Genoks Technology-Ankara. PCR reactions were performed in a total volume of 50µl containing 1.25 U Taq DNA polymerase, 2 µl- 5 µM MgCl<sub>2</sub>, 5 µl-10X Tris-HCl (Vivantis, PL 1202), 0.2mM of each deoxynucleoside triphosphates, and a specific primer pair. The concentration of each primer was 0.5 µM for all target bacteria. DNA amplification was performed in a thermal cycler (BIO-RAD, MJ Mini Personal Thermal Cycler). PCR products were stored at -80°C.

The amplification products were analyzed by 1.8% agarose gel electrophoresis containing 0.5% ethidium bromide in TBE buffer (Tris-borate EDTA) at 100 V for 1 h and visualized under ultraviolet light. The identity of each band was determined in comparison using a 100-bp DNA ladder.

#### Statistical analyses

Data collected for each case were recorded on an electronic spreadsheet and statistically analyzed by using SPSS 12.0 (SPSS Inc., Chicago, IL). The prevalence of the target bacterial species was recorded as the percentage of the cases examined.

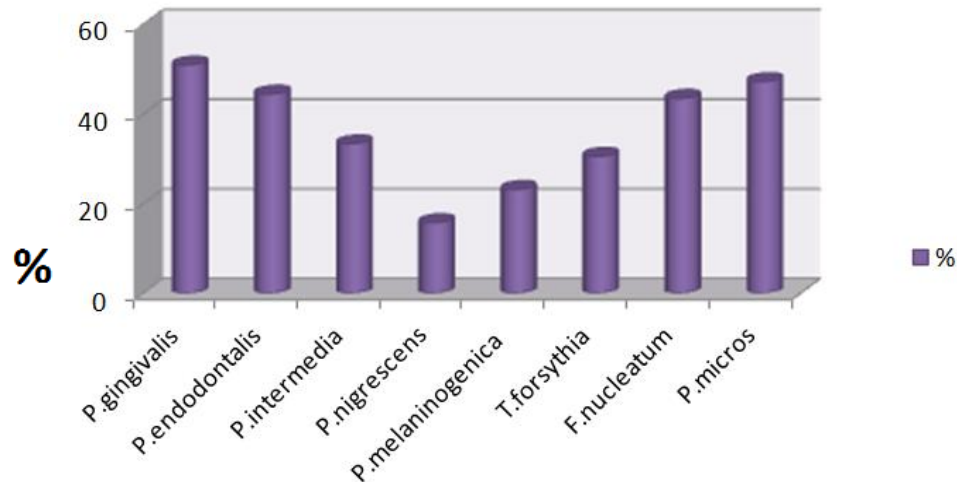
Descriptive statistical analyses were performed using the Pearson Chi-squared test.

#### RESULTS

None of the specimens revealed negative bacterial DNA that was amplified by using universal eubacterial primers. The incidence of detection of the selected bacterial species for all samples is summarized in Figure 1.

The results of NAAM analysis showed that all specimens were positive at least for 1 or more samples in the primary and secondary teeth infection. The most frequently detected bacteria in all specimens was *P. gingivalis*, followed successively by *P. micra*, *P. endodontalis*, *F. nucleatum*, *P. intermedia* and *T. forsythia* (Table 2). The percentage of all selected bacteria found in the primary infection group was higher than the secondary infection group except for *P. intermedia*. However, statistically significant difference was found only for *T. forsythia* and *F. nucleatum* ( $p < 0.05$ ), which were both higher in the primary infection compared to the secondary infection group.

In the primary infection group 46 out of 72 (63.9%) samples consisted three or more species per canal whereas this ratio was lower in the secondary infection



**Figure 1.** Incidence of bacteria in all specimens.

**Table 2.** The incidence of bacteria detected in the primary and secondary endodontic infections.

Target bacteria	Primary endodontic infection		Secondary endodontic infection		Failed endodontic treatment
		%		%	
<i>Porphyromonas gingivalis</i>	39/72	54.2	16/35	45.7	
<i>Porphyromonas endodontalis</i>	35/72	48.6	13/35	37.1	
<i>Prevotella intermedia</i>	22/72	30.6	14/35	40	
<i>Prevotella nigrescens</i>	12/72	16.7	5/35	14.3	
<i>Prevotella melaninogenica</i>	18/72	25	6/35	17.3	
<i>Tannerella forsythia</i>	28/72	38.9*	5/35	14.3	
<i>Fusobacterium nucleatum</i>	38/72	52.8†	9/35	25.7	
<i>Parvimonas micra</i>	35/72	48.6	15/35	42.9	

\*  $p < 0.008$  †  $p < 0.010$ .

**Table 3.** The incidence of bacterial combinations in specimens of the primary and secondary endodontic infections.

Bacterial combination	N	%
<i>P.intermedia</i> + <i>P.micra</i>	30/107	28
<i>F.nucleatum</i> + <i>P.micra</i>	28/107	26.2
<i>P.endodontalis</i> + <i>P.gingivalis</i>	26/107	24.3
<i>P.endodontalis</i> + <i>P.micra</i>	24/107	22.4
<i>T.forsythia</i> + <i>P.gingivalis</i>	23/107	21.5
<i>P.micra</i> + <i>P.gingivalis</i>	23/107	21.5
<i>F.nucleatum</i> + <i>P.gingivalis</i>	19/107	17.8
<i>T.forsythia</i> + <i>P.micra</i>	19/107	17.8
<i>F.nucleatum</i> + <i>P.intermedia</i>	16/107	15

group (34.3%) and this difference was statistically significant ( $p < 0.05$ ). As for the specimens containing only one targeted bacteria, the secondary infection group had

a higher incidence (31.4%) than the primary infection group (18.1%). However, there was no statistically significant difference between two groups ( $p > 0.05$ ).

In the present study, the combination of bacterial species were also investigated and *P.micra* was found together with the species *P.intermedia*, *F.nucleatum*, *P.endodontalis*, *P.gingivalis* and *T.forsythia* in all specimens with the incidences of 28, 26, 2, 22, 4, 21, 5 and 17, 8%, respectively. Table 3 shows the most prevalent bacterial combinations in all specimens tested.

Eighty-six of 107 (80%) samples had symptomatic root canal infections and the association was found between spontaneous pain and primary infection group (62/72). This ratio demonstrated a statistically significant ( $p < 0.05$ ) relationship between clinical signs and symptoms and the primary infections. The 60.5% of symptomatic cases harbored 3 or more of the tested endodontic pathogens, which could point out the relationship between bacterial complexity and clinical characterization of the infection ( $p < 0.05$ ). Table 4 shows the prevalence of micro-

**Table 4.** The prevalence of microorganisms associated with the clinical signs and symptoms of infected root canals.

Target microorganism	N		Pain	Previous pain	Tenderness to percussion	Swelling	Periapical radiolucency	Fistula
<i>P. gingivalis</i>	55	N	26	10	37§	32†	17	41
		%	47.3	18.2	67.3	58.2	30.9	74.5
<i>P. endodontalis</i>	48	N	22	10	24	18	13	28
		%	45.8	20.8	50	37.5	27.1	59.6
<i>P. intermedia</i>	36	N	19	5	16	14	11	28
		%	52.7	13.9	44.4	38.8	30.6	77.7
<i>P. nigrescens</i>	17	N	8	2	10	8	2	11
		%	47.05	11.8	58.8	47.1	11.8	64.70
<i>P. melaninogenia</i>	24	N	19*	3	14	14‡	11	19
		%	79.2	12.5	58.3	58.3	45.8	79.1
<i>T. forsythia</i>	33	N	11	7	18	14	14	22
		%	33.3	21.2	54.5	42.4	42.4	66.6
<i>F. nucleatum</i>	47	N	20	9	26	21	15	34
		%	42.6	19.1	55.3	44.7	31.9	72.3
<i>P. micra</i>	51	N	20	5	21	21	14	37
		%	40	10	42	42	28	74

\* p: 0.000, † p: 0.018, ‡ p: 0.022, § p: 0.001.

organisms associated with clinical signs and symptoms in 107 infected root canal samples. There was a significant association between tenderness to percussion and *P. gingivalis* ( $p < 0.05$ ), pain with *P. melaninogenica* ( $p < 0.05$ ) and swelling with both *P. gingivalis* ( $p < 0.05$ ) and *P. melaninogenica* ( $p < 0.05$ ).

## DISCUSSION

The purpose of this study was to evaluate the presence of selected bacterial pathogens by using both universal and specific PCR primers in the primary and secondary root canal samples and to associate these species with clinical signs and symptoms. In this study *P. gingivalis* was the most frequently detected bacteria in all specimens both from patients with primary and secondary root canal infections. The black-pigmented species studied were detected at a higher frequency in teeth with necrotic pulp than in teeth with failing endodontic treatment. In accordance with this study, Gomes et al. (2005) reported that *P. gingivalis*, *P. endodontalis*, *P. intermedia* and *P. nigrescens* were detected more frequently in untreated teeth with necrotic pulp than in teeth with failing endodontic treatment. Blome et al. (2008) reported *P. endodontalis* as the prevalent microorganisms in primary and secondary endodontic infections but in contrast with our finding

they found *P. gingivalis* in low ratios. *P. endodontalis* has been almost particularly associated with endodontic infections, and its pathogenicity depends on the presence of the other species in a consortium (van Winkelhoff et al., 1992).

*F. nucleatum* was found as the second prevalent bacteria in primary endodontic group and also was found in low ratios in secondary infection group. *F. nucleatum* was previously shown to increase pathogenicities of other organisms in mixed culture, especially those of *P. gingivalis* and *P. intermedia* (Baumgartner and Falker, 1991, Siqueira et al., 2000). *F. nucleatum* and *P. gingivalis* have also been described as common endodontic bacterial pathogens in other study. (Podbielski et al., 2003). In accordance with our study, Vianna et al. detected *F. nucleatum*, *T. forsythia*, *P. gingivalis* frequently in necrotic root canal by the DNA chip (Vianna et al., 2005).

Fouad et al. (2002) demonstrated that *P. nigrescens* is more prevalent in endodontic infections than *P. intermedia*. In contrast, in our study *P. intermedia* ratio is higher than *P. nigrescens*. Tomazinho and Avila-Campos (2007) found that *P. gingivalis* and *P. nigrescens* were the most prevalent, followed by *P. intermedia* and *P. endodontalis* in 60 PCR samples taken from chronic endodontic infections. *P. endodontalis* and *P. gingivalis* have been consistently encountered in endodontic infections, and attributed a role for both in the etiology of acute abscess



(van Winkelhoff et al., 1985; Sundqvist et al., 1989).

*T. forsythia* had never been detected in root canals by culture but is confirmed that this organism is a common member of the microbiota associated with different types of primary infections including abscess by molecular biology approaches (Fouad et al., 2002; Siqueira and Roças, 2003). In this study, it is of interest that a gram negative rod, *T. Forsythia*, was detected in 33 of 107 root canal samples and that in 30 of these 33 samples, this bacterium was always associated with one or more members of the black pigmented gram negative rods. In accordance with this study, *T. forsythia* was found in root canals from 40 to 59.1% and this species is suggested to play a major role in the pathogenicity of primary endodontic infections (Siqueria and Roças, 2003; Blome et al., 2008).

Bacterial combinations in root canals may be more pathogenic than individual strains (Fabricus et al., 1982). Therefore, it is important to determine the association of bacterial combinations with clinical signs and symptoms or treatment outcome, as well as the association of certain microorganisms with each other. In accordance with some previous studies reporting the positive ecological relationship between *P. gingivalis* and *T. forsythia*, these two bacteria were found together in 23 of 107 samples in our study (Jung et al. 2000, Roças et al., 2001). Moreover, in 30 of 107 endodontic samples *P. intermedia* and *P. micra* combination was detected. In all tested specimens, *P. micra* had higher combination ratios compared to the other anaerobic species. *P. micra* is also detected in 28 of 107 samples with *F. nucleatum*, that is concordant with the results reported by Blome et al. (2008) but further research is needed to confirm these bacterial relations.

Our results demonstrated that *P. gingivalis* was associated with symptoms of tenderness to percussion and swelling. Jacinto et al. (2003) found a relation between pain on palpation and *P. gingivalis* and *Peptostreptococcus* spp. And similar to our study Siqueira et al. (2000) found six bacterial species which were detected in teeth tender to percussion, of which *P. gingivalis* and *P. micra* were the predominant species.

The disparity in the composition of root canal microbiota can be related to sampling method, different methodological techniques, and other factors including geographical effects (Dumani et al., 2012). Baumgartner et al. (2004) used PCR to detect the presence of selected bacterial species in samples of acute periodontal abscesses collected from the United States and Brazil. They found that the prevalence of *P. intermedia*, *P. nigrescens*, *P. tanneriae*, *F. nucleatum*, *P. gingivalis* markedly differed in two locations.

Apical periodontitis has a polymicrobial etiology and the functional role of species in this mixed endodontic consortium needs further efforts directed towards finding associations between them and clinical symptoms and conditions. It is claimed that, whether it is a failed endo-

dontic treatment or a necrotic pulp space, the environment selects for microorganisms that possess traits suited to establishing and sustaining the disease process (Figdor and Sundqvist, 2007).

## Conclusion

Findings indicated that the prevalence of some species found in the primary infection group were higher than in the secondary infection group. In this study there was a significant association between tenderness to percussion and *P. gingivalis*, pain with *P. melaninogenica* and swelling with both *P. gingivalis* and *P. melaninogenica*.

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