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Full Length Research Paper

Screening and evaluation of herbicidal metabolites produced by *Trichoderma* spp.

Wenfeng Kuang*, Chengfan Wang and Weili Mao

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Laboratory bioassays were conducted to screen and evaluate the potential herbicidal metabolites produced by *Trichoderma.longibrachiatum* (Tr673), *T. harzianum* (Tr319), *T. viride* (Tr347) *T. koningii* (Tr324) and *T. asperellum* (Tr85) on inhibiting seed germination (SGe) and shoot/root growth (SGr/RGr) of seedlings of *Porslane (Portulaca oleracea), Barnyardgrass (Echinochloa crus-galli), and Amaranthus retroflexus L*. The culture filtrates generated from fermented liquids with each of the *Trichoderma* strains were diluted into 10 and 20% by adding sterilized water, which was then applied onto the weed seeds individually. The IR (%) of SGr/RGr of seedlings were significantly higher (*P*≤ 0.05) in the treatments with Tr673 culture filtrate generated from potato dextrose broth (PDB) for all treated weed seeds compared to those in the treatments generated from other culture media with Tr673 or Tr347. The IR (%) on SGe and SGr/RGr of seedlings in the treatment with Tr673 culture filtrate generated from PDB + 0.4% sodium glutamate were significantly higher (*P*≤ 0.05) than those generated from PDB alone. The result of the bioassay with the crude extract (62.5 mg·L^{-1}) generated from Tr673 culture filtrate showed a similar effect on decreasing the SGr/RGr rates (%) of the weeds and cucumber compared to those with the chemical herbicide-glyphosate.

Key words: *Trichoderma,* herbicidal activity, metabolites, inhibiting, crude extract.

INTRODUCTION

About 842 million people or around 14% of the world's population suffered from chronic hunger due to not getting enough food to conduct an active life from 2011 to 2013 (FAO, 2013). Weed is one of the most serious causes of economic losses in agricultural production. Losses of agriculture caused by weeds are about 5 to 10% in developed countries, while losses can be up to 20 to 30% in developing or emerging countries (FAO, 2006). *Portulaca oleracea, Amaranthus retroflexus* and *Echinochloa crus-galli* are the world's worst weeds (Holm et al., 1991).

Due to lack of labor for weeding and other economic reasons, the use of chemical herbicides has been increased and herbicides have made great contribution for farming on weed control and increasing yields of food production. However, it has become a serious

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problem that repeated use of the same chemicals, or chemicals with the same mode of action would lead to the selection and buildup of resistant pest populations (Carey et al., 1995; Daou and Talbert, 1999). We are facing the challenges on weed control with synthetic agro-chemicals such as emergence of weeds resistant to herbicides (Yuan et al., 2007; Llewellyn et al., 2009), and concerns about the side effects caused by herbicides in food, soil, ground water and atmosphere (Rial-Otero et al., 2005).

The use of microbes for pest management is one of the most effective biocontrol strategies in agriculture (Ahmad et al., 2011). Natural herbicides are eco-friendly, biodegradable, and less toxic to plants and beneficial microorganisms. They are biosynthesized through specialized pathways and exhibit a wide range of biocontrol activities (Hanson, 2003). Fungi are known to produce diverse active metabolites including herbicidal metabolites (Saxena and Pandey, 2001). It has been one of the most important research areas in weed management to screen and evaluate herbicidal metabolites from fungal products. Fungi such as *Alternaria, Fusarium, Coletotrichum*, etc. can produce phytotoxin (Andolfi et al., 2005; Pedras et al., 2009; Junko and Kenji, 1995). Culture medium is an important influence factor of herbicidal activities, and different carbon and nitrogen sources have different effects on the herbicidal activity of these fungi (Eduardo et al., 2013).

Trichoderma may act as symbionts of plants and have been studied as biopesticides and biofertilizers due to their abilities to protect crops from weeds and promote vegetative growth (Hill et al., 1995; Harman, 2000, 2006). *Trichoderma* spp. is a well-known producers of secondary metabolites with different biological activities, not only potential antibiotics, but also mycotoxins and more than 100 metabolites with antibiotic activities such as pyrones, terpenes and metabolites derived from amino acids and polypeptides (Leclerc et al., 1998; Mukherjee et al., 2012). However, *Trichoderma* products for weed control are still commercially limited and few known studies in this area are restricted to *T. virens* only (Hutchinson, 1999; Heraux et al., 2005 a, b). In this study, we designed to screen and evaluate the herbicidal activities of the culture filtrates produced by *Trichoderma longibrachiatum* (Tr673), *T. harzianum* (Tr319), *T. viride* (Tr347), *T. koningii* (Tr324) and *T. asperellum* (Tr85).

MATERIALS AND METHODS

Trichoderma strains and seeds of weeds and cucumber

The strains of Tr673 (*T. longibrachiatum*), Tr319 (*T. harzianum*), Tr347 (*T. viride*), Tr324 (*T. koningii*) and Tr85 (*T. asperellum*) used in this study were provided by Shanghai WanLiHua BioTech Company. Unless otherwise noted, all the strains were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit) at 4°C. Weed seeds of *P. oleracea, A. retroflexus* L and *B. campestris* L. and cucumber (BiYu 2) were purchased from agricultural markets.

Medium and stock solution

1. Potato Dextrose Broth (PDB, $g{\cdot}L^{-1}$): 20 potato-powder, 20 dextrose, 3 K_2HPO_4, 1.5 $MgSO_4$ and 0.005 to 0.01 VB_1).
2. Molasses yeast-extract medium (MYM, $g{\cdot}L^{-1}$): 30 molasses, 10 yeast extract.
3. Synthetic medium (SM, $g{\cdot}L^{-1}$): $MgSO_4$ 0.2; KCL 0.15, KH_2PO_4 0.9; NH_4NO_3 3.0; dextrose
4. Sabouraud's dextrose broth (SDB, $g{\cdot}L^{-1}$): Dextrose 40, mycological peptone 10.
5. Stock solution of glyphosate (62.5 $mg{\cdot}L^{-1}$): 30% glyphosate aqueous solution (Wynca Group) glyphosate 62.5 µl and SDW 300 ml.

Preparation of culture filtrates

Plugs (5-mm) of each *Trichoderma* strain collected from actively growing margins of PDA cultures were transferred into 1.0 L flasks containing 200 ml of PDB, MYM, SM and SDB. Inoculated flasks were put onto a shaking incubator at 28°C and 200 r/min for 6 days. The culture filtrate was individually filtrated and the pH of the culture filtrates were adjusted to 7.0 by adding 0.5 $mol{\cdot}L^{-1}$ NaOH or 0.5 $mol{\cdot}L^{-1}$ HCl. To obtain the cell free supernatants, the culture filtrates were set on a centrifuge at 10,000×g for 15 min at 4ºC and then passed through a 0.2 µm filter (Sartorius) separately. The 10 and 20% diluted culture filtrates were made by adding SDW.

Laboratory bioassays

Screening of herbicidal metabolites generated from Trichoderma culture filtrates

To screen the herbicidal activities of the culture filtrates generated from each of the *Trichoderma* strains, *in vitro* bioassays were conducted according to the filter paper method described by Deba et al. (2007) and Xu et al. (2009). Seeds of *P. oleracea, A. retroflexus* L, and *B. campestris* L were immersed in 1% sodium hypochlorite for 10 min followed by 3 times of washings with SDW for surface sterilization. A total of 20 seeds of each of the weeds were placed into Petri plates (9-cm) with two layers of filter papers (Whatman No. 1) on the bottom. For the test of inhibiting seed germination (SGe), the filter papers were wetted with 5 ml of 10 and 20% of each of the culture filtrates, respectively. For the control treatment, the filter papers were wetted with 5 ml of sterilized PDB.

Each of the treatments has 3 replicates. The Petri dishes with treated seeds were put into a growth chamber at 25°C with a 12-h photoperiod for 7 days. Data of inhibiting rate (IR, %) of SGe was recorded for the total viable seeds.

The IR (%) of SGe was calculated as follows:

$$\text{The IR (\%) of SGe} = 100 - [(N^*\text{-treatment}/N\text{-control})\times 100] \quad (1)$$

Where N*-treatment is the number of germinated seeds of different treatments; N-control is the number of germinated seeds in the control.

For the test of inhibition shoot/root growth (SGr/RGr) of seedlings, the filter papers of all Petri dishes were wetted with 5 ml of SDW first, after all the seeds geminated, the filter papers were again wetted with 5 ml of 10 and 20% of each of the culture filtrates, respectively. For the control treatments of chemical and

blank, 5 ml of glyphosate stock solution and SDW were added, respectively. Data of IR (%) of SGr/RGr of seedlings was collected 5 days after the culture filtrates were applied.

The IR (%) of SGr/RGr of seedlings was calculated as follows:

The IR (%) of SGr/RGr of seedlings = 128100 - [(L*-treatment/l-control)×100] (2)

L*-treatment is the average of the root/shoot length of 15 seedlings picked randomly from each of the Petri dishes of different treatments. L-control is the average of the root/shoot length of 15 seedlings picked randomly from each of the Petri dishes of control treatment.

Screening of a culture medium for optimal production of herbicidal metabolites

In order to screen a medium for optimal production of herbicidal metabolites, PDB, MYM, SM and SDB were prepared and inoculated with Tr673 and Tr347. As described previously under the preparation of culture filtrates, 10% diluted culture filtrates generated from each of the growth media were separately prepared and applied onto the seeds of *A. retroflexus.* Data of IR (%) of SGr/RGr of seedlings was recorded 5 days after the seeds were treated.

For optimal production of herbicidal metabolites, a single-variable optimization strategy utilized to determine the C-source and N-source in the culture media with Tr673. To screen the C- source, 2% of soluble starch, sucrose, lactose, glycerol, xylose or maltose were separately used to replace the glucose in original PDB. After finalizing the C-source, 0.4% of amino acids of tryptone, arginine, glycine, sodium glutamate, urea or aspartic acid were separately added into PDB with a selected C-source. As described previously under the preparation of culture filtrates, 10% diluted culture filtrate was separately prepared and applied onto the seeds of *A. retroflexus* L. Data of IR (%) of SGr/RGr of seedlings were recorded 5 days after the seeds were treated.

Test of the herbicidal activity with Tr673 crude extract

The crude extract of Tr673 was obtained by utilizing macroporous adsorption method. The conditions for adsorption of macroporous adsorption resin HZ806 (Shanghai Huazhen Sci. & Tech. Co., Ltd) were a processing of volume as 32 bed volumes (BV), pH value of 4, and flow rate of 2 BV/h; and those for desorption of HZ806 were a 50:50 (*v*/*v*) ratio of ethanol to water. The crude extracts were finally obtained by adding 50% of ethanol elution and a process of vacuum distillation.

The dried crude extract (5 g) was dissolved with 10 ml of acetone/water (50:50, *v*/*v*) and the solution was then diluted into the concentrations of 7.8, 15.6, 31.3, 62.5, 125 and 250 $mg.mL^{-1}$ with appropriate amount of SDW. Sterilization of the extracts was made by passing the solutions separately through a 0.2 μm filter (Sartorius). As described previously under screening of herbicidal metabolites generated from Trichoderma culture filtrates, an *in vitro* bioassay was conducted with the weeds of *P. oleracea, E. crus-galli, A. retroflexus* L, *B. campestris* L. and cucumber, 5 ml of each of diluted crude extract solutions and stock solution of glyphosate were added into each of the correct Petri dishes. Five days after seed treating, the data were collected.

Statistical analysis

A completely randomized design was used for all of the bioassays and the data was analyzed by ANOVA (analysis of variance). The common difference of the treatments was considered to be significant at the 5% level ($P \leq 0.05$).

RESULTS

Screening of herbicidal metabolites with *Trichoderma* culture filtrates

The results of the bioassay for screening and evaluation of the herbicidal metabolites (Table 1) showed that there were no significant differences on the inhibitory rates (IR%) of seed germination (SGe) among the treatments with 10 and 20%, respectively of diluted culture filtrates generated from the strains ofTr324, Tr85, Tr319, Tr347 and the SDW control, but the parameters of the IR (%) of SGe in each of these treatments were significantly lower ($P \leq 0.05$) than those in the treatment with the strain of Tr673. The IR (%) of shoot/root growth (SGr/RGr) of seedlings in the treatment with 10 and 20%, respectively of diluted culture filtrates generated from the strains of Tr347 were significantly higher ($P \leq 0.05$) than those in the treatments with the strains of Tr324, Tr85 and Tr319, but the parameters of the IR (%) of SGr/RGr of seedlings in each of these treatments were significantly lower ($P \leq 0.05$) than those in the treatment with the strain of Tr673.

Screening of a culture medium for optimal production of herbicidal metabolites

To screen a culture medium for optimal of herbicidal metabolites, 4 media of PDB, PYM, SM and SDB were tested. The results indicated (Figure 1) that the IR (%) of SGr/RGr of seedlings were significantly higher ($P \leq 0.05$) in the treatments with the culture mediainoculated with Tr673 for all treated weed seeds compared to those inoculated with Tr347, except in the treatments with the culture media of SM on *A. retroflexus*; SM, MYM, SDB on *E. crusgalli*; SM and MYM on *P. oleracea* for SGr, and MYM on *A. retroflexus*; SM, MYM on *E. crusgalli*; SM on *P. oleracea* and SM on *B. campestris* for RGr. There were no significant differences on the IR (%) of SGr/RGr of seedlings between each of the culture media inoculated with Tr673 and Tr347. The IR (%) of SGr/RGr of seedlings were significantly higher ($P \leq 0.05$) in the treatments with PDA inoculated with Tr673 for all treated weed seeds compared to those in the treatments with other culture media inoculated with Tr673 or Tr347. The results of determining the C-source with different culture media showed (Figure 2) that there were no significant differences between the treatments with the C-sources of glucose (original PDB) and sucrose on the IR (%) of SGr of seedlings, but the parameters of the IR (%) of SGr in each of the two treatments were significantly higher ($P \leq 0.05$) than those with each of other C-sources.

Table 1. Effect of herbicidal metabolites generated from *Trichoderma* culture filtrates on inhabiting SGe and RGr/SGr of seedlings of *A. retroflexus*, *P. oleracea* and *B. campestris* L.

Treatment		Inhibitory rate (%)								
		A. retroflexus			*P.oleracea*			*B. campestris* L.		
Strains	**C*(%)**	**SGe**	**RGr**	**SGr**	**SGe**	**RGr**	**SGr**	**SGe**	**RGr**	**SGr**
Blank PDB (Ctrl.)	20	3^{c}	-1.88^{ef}	-1.65^{de}	2.0^{c}	-4.03^{de}	$-.56^{d}$	13^{b}	-1.79^{e}	-4.30^{e}
Tr347-*T. viride*	10	12^{c}	4.38^{d}	2.16^{d}	2.0^{c}	4.40^{c}	4.27^{cd}	13^{b}	32.51^{c}	41.47^{c}
	20	25^{b}	12.69^{c}	10.55^{c}	2.0^{c}	9.19^{c}	8.84^{c}	16^{b}	14.27^{d}	8.39^{d}
Tr324-*T. koningii*	10	10^{c}	-0.12^{de}	1.52^{d}	2.0^{c}	3.27^{cd}	4.38^{cd}	12^{b}	0.56^{e}	-2.57^{e}
	20	9^{c}	0.81^{de}	-3.75^{de}	2.0^{c}	5.50^{c}	5.14^{cd}	14^{b}	$.69^{e}$	1.02^{de}
Tr85-*T. asperellum*	10	4^{c}	-6.11^{f}	-28.15^{f}	2.0^{c}	-5.16^{e}	-7.36^{e}	16^{b}	-2.83^{e}	-4.95^{e}
	20	7^{c}	2.5^{ed}	-5.01^{de}	2.0^{c}	3.51^{cd}	-0.60^{d}	16^{b}	-0.37^{e}	-4.56^{e}
Tr319-*T. harzianum*	10	9^{c}	0.02^{de}	-2.03^{de}	2.0^{c}	4.45^{c}	1.20^{d}	17^{b}	-0.95^{e}	-3.95^{e}
	20	4^{c}	-0.17^{de}	$.25^{de}$	2.0^{c}	5.45^{c}	3.62^{cd}	16^{b}	-0.49^{e}	-7.36^{e}
Tr673-*T .longibrachiatum*	10	26^{b}	34.8^{b}	22.93^{b}	12^{b}	30.10^{b}	30.95^{b}	18^{b}	62.74^{b}	60.83^{b}
	20	46^{a}	80.5^{a}	71.91^{a}	48^{a}	71.31^{a}	68.63^{a}	46^{a}	77.68^{a}	76.75^{a}

According to Duncan's Multiple Range Test (P ≤ 0.05, n= 5), means with the same letter among the treatments are not significantly different from each other. C*: Concentration.

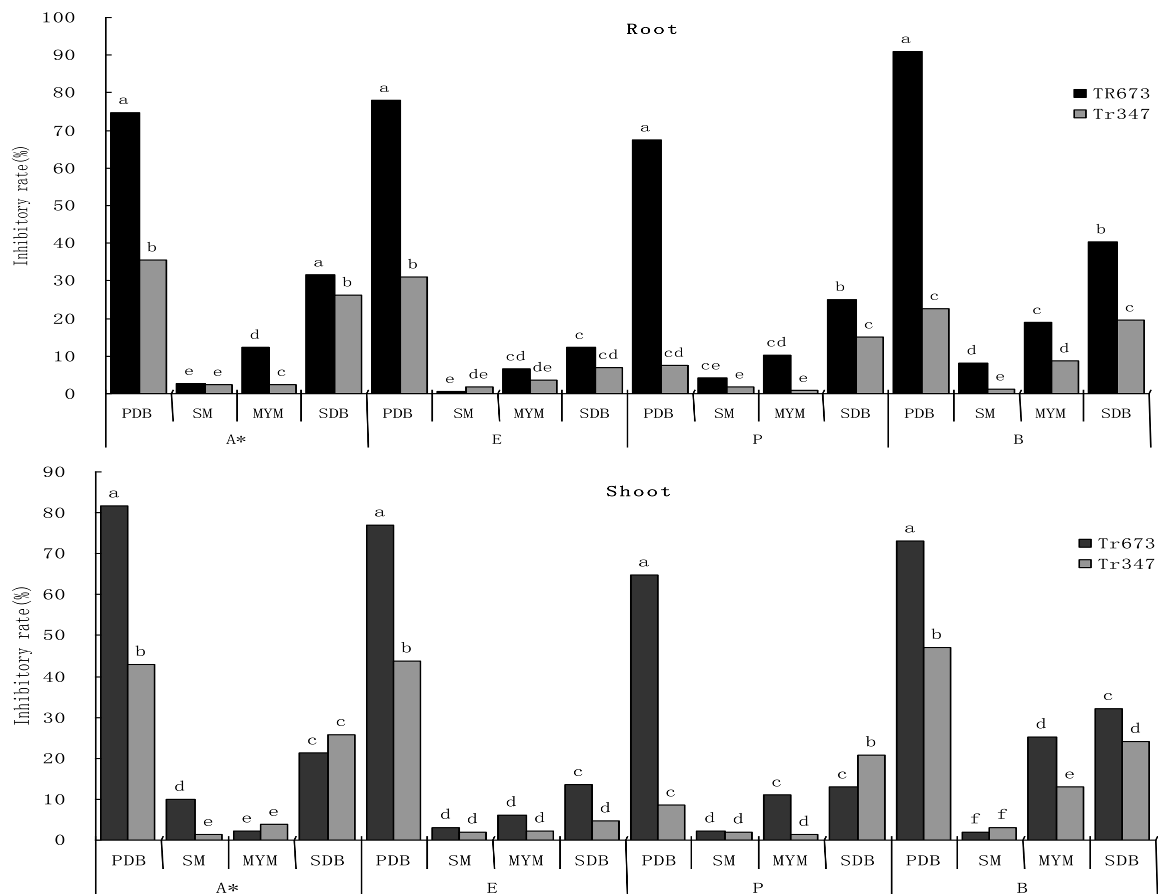

Figure 1. Effect of herbicidal metabolites generated from Tr673 and Tr347 in different culture media on inhabiting root/shoot growth of seedlings. According to Duncan's Multiple Range Test ($P \leq 0.05$, n=5), means with the same letter among the treatments are not significantly different from each other. *: A, *A. retroflexus,* E, *E. crusgalli, P, P. oleracea,* B, *B. campestris.*

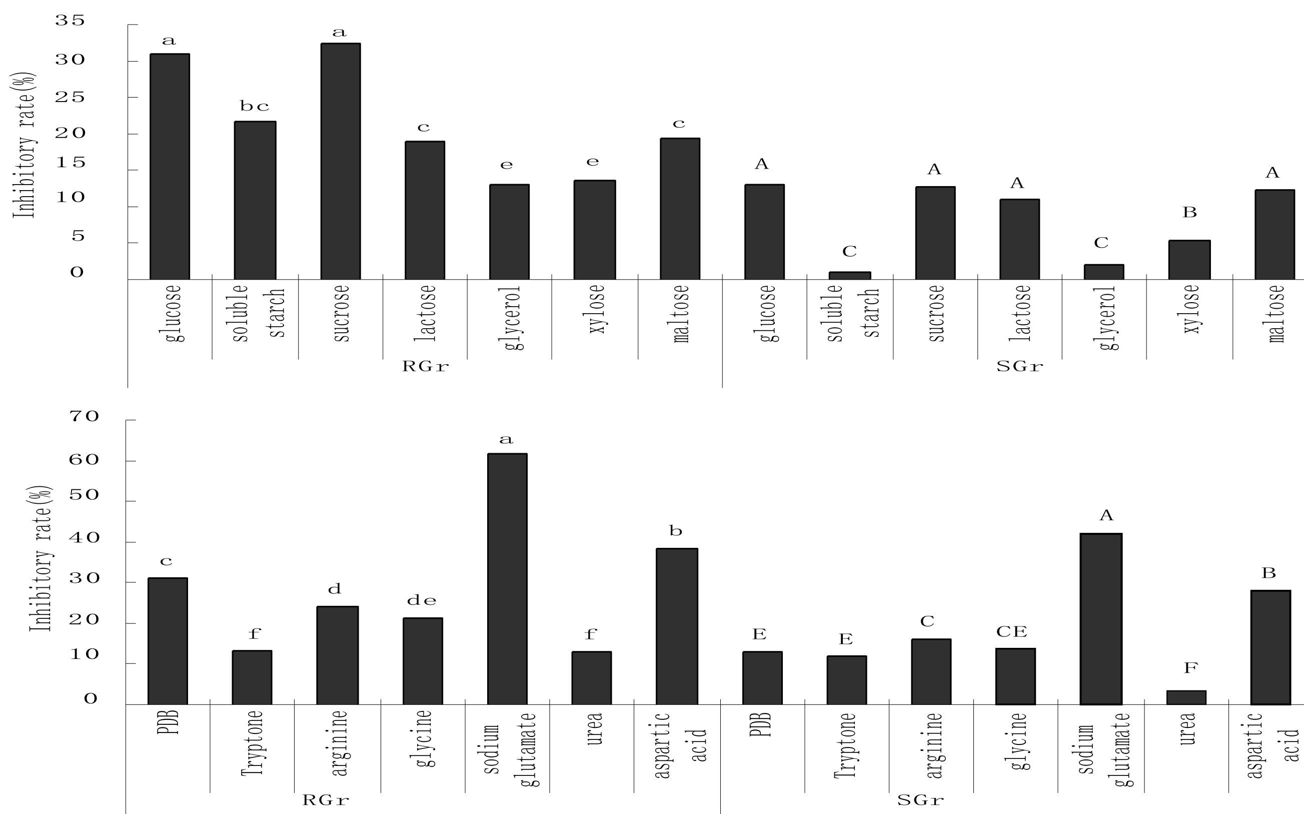

Figure 2. Effect of herbicidal metabolites generated from Tr673 culture filtrates of PDB with different C-sources and N-sources on inhabiting RGr/SGr of seedlings. According to Duncan's Multiple Range Test (P≤ 0.05, n= 5), means with the same letter among the treatments are not significantly different from each other different from each other.

Similarly, there were no significant differences among the treatments with the C-sources of glucose, sucrose, lactose and maltose on the IR (%) of RGr of seedlings, but the parameters of the IR (%) of RGr in these four treatments were significantly higher (*P ≤ 0.05*) than those with soluble starch, glycerol or maltose. The results indicated that glucose is one of the best C-sources used in the culture medium of PDB for optimal production of herbicidal metabolites.

For the test of determining the N-sources added into PDB, the results showed (Figure 2) that 4% of sodium glutamate is the best N-source added into PDB to increase the production of herbicidal metabolites, it had a significantly higher (*P≤ 0.05*) IR (%) on both SGr and RGr of seedlings than any of other individual N-source added into PDB or PDB alone.

Test of the herbicidal activity with Tr673 crude extract

The results of the test with Tr673 crude extract showed that the IR (%) of SGr/RGr of seedlings was significantly higher (*P* ≤ 0.05) in the treatment with diluted solution at the concentration of 125 $mg{\cdot}L^{-1}$ than those in the treatments with diluted solutions at lower concentrations of 7.8, 15.6, 31.3 and 62.5 $mg{\cdot}L^{-1}$ and the stock solution of glyphosate, but the parameters of the IR (%) of SGr/RGr of seedlings in this treatment were significantly lower (*P ≤ 0.05*) than those in the treatments with diluted solutions at higher concentrations of 250 and 500 $mg{\cdot}l^{-1}$ (Table 2).

DISCUSSION

As a biocontrol agent, *Trichoderma* has been extensively used in agriculture worldwide for crop protection due to its antimicrobial activities on suppressing a broad range of phytopathogens (Harman, 2000, 2006). The diverse type of secondary metabolites including gliovirin, gliotoxin, viridian, and viridiol produced by *Trichoderma* spp. has also been demonstrated on pest control (Jones and Hancock, 1987). Harziphilone and fleephilone were used as HIV, regulation of virion expression/Rev response element (RRE) binding inhibitors produced by T. harzianum (Qian-Cutrone et al., 1996). The volatile compound of 6-pentyl-2H-pyran-2-one produced by *T. harzianum* is active to

Table 2. Effect of herbicidal activity of Tr673 crude extract on inhibiting RGr/SGr of seedlings.

Treatment*	Inhibitory rate (%)									
	P. oleracea		***E. crus-galli***		***A. retroflexus* L**		***B. campestris* L**		***Cucumber***	
	SGr	**RGr**	**SGr**	**RGr**	**SGr**	**RGr**	**SGr**	**RGr**	**SGr**	**RGr**
500	100.0^{a}	100.0^{a}	98.7^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}
250	94.3^{b}	100.0^{a}	74.3^{b}	100.0^{a}	97.0^{b}	100.0^{a}	100.0^{a}	100.0^{a}	87.7^{b}	100.0^{a}
125	70.7^{c}	87.7^{b}	56.0^{d}	88.0^{b}	82.3^{c}	83.7^{b}	93.0^{b}	92.3^{b}	66.0^{c}	86.3^{b}
62.5	52.0^{d}	65.7^{c}	38.7^{e}	66.0^{d}	44.3^{e}	50.7^{c}	55.0^{c}	78.3^{c}	40.0^{d}	59.7^{c}
31.3	34.3^{f}	40.3^{e}	9.7^{f}	36.0^{e}	25.0^{f}	28.7^{d}	28.7^{d}	47.7^{e}	9.3^{e}	32.7^{d}
15.7	0.0g	10.7^{f}	-0.7g	-0.7^{f}	1.0g	1.0^{e}	1.3^{e}	14.3^{f}	1.7^{f}	10.3^{e}
7.8	-2g	0.7g	-1.3g	-0.3^{f}	0.0g	0.0^{e}	2.0^{e}	-0.7g	-2.7 ^{f}	1.3^{f}
Glyphosate**	44^{e}	49.7^{d}	63.39^{c}	74.7^{c}	53.0 ^{d}	49.7^{c}	54.3^{c}	70.3^{d}	39.3^{d}	61.0^{c}

According to *Duncan's* Multiple Range Test ($P \leq 0.05$, n= 5), means with the same letter among the treatments are not significantly different from each other. *, sample concentration at $mg \cdot L^{-1}$; **, in a concentration at 62.5 $mg \cdot L^{-1}$.

suppress *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici* (Scarselletti and Faull, 1994) and viridiol is strongly phytotoxic (Jones and Hancock, 1987). Chicken manure composted with *T. virens* exhibited its herbicidal activity on inhibiting the growth of *Setaria viridis* and *A. retroflexus* in the fields of rye cover crop (Heraux et al., 2005a). In this study, the culture filtrates generated from the strains of TR673 and Tr347exhibited strong herbicidal activities on inhibiting SG and RGr/SGr of seedlings of tested weeds. These findings are in certain agreement with the results of the studies conducted by Hutchinson (1999) and Héraux et al. (2005a, b).

However, it was the first time to report the observation that the culture filtrate produced by Tr673, a strain of *T. longibrachiatum*, exhibited a stronger herbicidal effect on suppressing the growth of tested weeds compared to other *Trichoderma* strains. It also indicates that *T. longibrachiatum* might produce herbicidal compounds, which will be detected in the subsequent studies.

Nusrat et al. (2013) observed that the maximum biomass of *T. harzianum* was produced in PDB, but the minimum was produced in water broth. Harman et al. (1991) reported that maximum fresh and dry weight of biomass was obtained during fermentation process with PDB followed by TSM, Czapek-Dox and MEB. In present study, it was observed that Tr673 culture filtrate generated with PDB showed a significant herbicidal effect on inhibiting RGr/SGr tested weed seedlings compared to those with MYM, SM and SDB. It indicated that PDB is one of the most suitable media for *Trichoderma* spp. to produce secondary metabolites with anti-pest activities. The role of carbon sources added in culture media is to provide energy for the growth of microorganisms and the basic carbon skeleton for microorganisms to synthesize secondary metabolites (Demain, 1986). Glucose and sucrose are monosaccharides and disaccharides respectively, which can be utilized by microorganisms for cell growth and in the processes of metabolisms (Demain, 1986). Leclerc et al. (1998) found that glucose is superior to sucrose as a growth-stimulating carbon source to be utilized by *Trichoderma* strains. They also found that when adding glutamic acid to the culture medium with *T. longibrachiatum*, the production of acidic peptides can be increased. In this study, we found that when 0.4% sodium glutamate was added to the culture medium, the herbicidal activity of Tr673 culture filtrate was markedly increased, which revealed that sodium glutamate might be one of important precursors of the herbicidal metabolites.

It is important to know the characteristics of chemical targets during the process of macroporous adsorption, which include polarity and molecular weight of the targets and the resin properties of polarity, surface area and diameter of pore. Non-polar resins can easily absorb non-polar substances in polar solvents, while polar resins can absorb polar targets from non-polar solvents (Zhang et al., 2011). We found in this study that actively herbicidal metabolites can be collected and enriched since HZ 806 have a large surface area and is matched to the target herbicidal metabolites in polarity. The result of the bioassay indicated that the crude extract had the same herbicidal effect on inhibiting SG and RGr/SGr of the weeds compared with Tr673 culture filtrate and the chemical herbicide of glyphosate.

Trichoderma metabolites, such as IAA and 6 - PP, can significantly promote the growth of the plant in moderate concentration (Vinale et al., 2008). Harman (2006) reported that *Trichoderma* spp. can decrease wilt incidence in chickpea plants and increase root development in numerous of other plants.

Narasimha et al. (2013) reported that seed treatment with *T. asperellum* enhanced the vigour index of tomato seedlings. In this study, we observed that the treatment

with Tr85 culture filtrate did not exhibit herbicidal effect on inhibiting SG and RGr/SGr of the weeds, however it showed a significant effect on increasing SGr of *A. retroflexus.* This indicated that Tr85 might be considered as a strain for plant growth promotion. Nowadays, the genus of *Trichoderma* has a great number of fungal strains that act as biocontrol agents, the antagonistic properties of which are based on the activation of multiple mechanisms (Benitez et al., 2004). The findings described in this study could be considered as a basic knowledge and references for the further studies in this area.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Nutritional characteristics of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria

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Due to the search for viable and cost-effective ways to provide nitrogen for forage grasses, inoculation with diazotrophs presents high potential as an alternative to plant nutrition by reducing the use of nitrogen fertilizers. The objective of this work was to evaluate the nutrition of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria grown in Cerrado soil (Brazilian Savanna). The experiment was conducted in a greenhouse with a completely randomized design using five treatments (three strains of associative nitrogen fixing bacteria [MTH2, MTB1 and Y2], an inoculant formed by combination of strains AbV5 and AbV6 (*Azospirillum brasilense*) and a control (100 mg dm^{-3} of N-urea]) and five replications, totaling 25 experimental units. The inoculation was performed by inserting a 5 mL aliquot of bacterial broth containing 10^9 cells mL^{-1} in the soil near the root system of each plant. Three cuts were made at 30, 60 and 90 days after sowing. The variables: Falker chlorophyll index, nitrogen concentration in shoots and roots and crude protein in shoots of marandu grass were evaluated. The highest values for Falker chlorophyll index, nitrogen concentration, and crude protein were observed in the three cuts in plants that received N fertilization. The MTH2 strain positively influenced the nitrogen concentration in shoots and roots, and crude protein in shoots in the first and second cuts of marandu grass, whereas in the third cut, these parameters were influenced by nitrogen fertilization. In conclusion, the inoculation with associative diazotrophic bacteria may contribute positively to the nitrogen nutrition of marandu grass.

Key words: *Brachiaria brizantha*, Cerrado, diazotrophic microorganisms.

INTRODUCTION

The genus *Brachiaria* (*Urochloa* spp.) represents a milestone in Brazilian cattle breeding and occupies large areas of the Cerrado Biome (Brazilian Savanna) in central Brazil. The choice of species and cultivars was informed by desirable characteristics such as forage plants (Fagundes et al., 2006). Currently, the species of

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greatest importance are *Brachiaria decumbens*, *Brachiaria brizantha*, *Brachiaria ruziziensis* and *Brachiaria humidicola* (Sobrinho et al., 2010).

Extensive areas of Brazil are occupied by pastures; some of these areas are already degraded while some are in the degradation stage (Guarda and Guarda, 2014). Brazil has about 180 million hectares cultivated with pasture, and degradation affects approximately 70% of these areas (Hungria et al., 2016). This degradation is a direct consequence of inadequate nutritional management of these pastures; it has caused decrease in soil fertility, and making agriculture sustainability a challenge (Fonte et al., 2014; Lai et al., 2016).

Although the use of nitrogen fertilizers contributes to the increase in pasture production (Boddey et al., 2000; Palmer et al., 2014), their use have limited efficacy because of the complex steps in the nitrogen cycle which occurs in the soil, and which can result in the loss of the fertilizer after its application (Van Groenigen et al., 2015).

Among the factors that lead to the degradation of pastures, nitrogen limitation is considered one of the most important, since nitrogen is the major contributor to the quality and production of pastures (Boddey et al., 2004). However, the low economic return and environmental impacts caused by fertilizer use have raised the need to recover the pastures by sustainable means (Vaio et al., 2008).

Currently, sustainable approaches are used in the entire world in order to minimize degradation impacts and improve the production in the pasture systems (Fonte et al., 2014). Thus, the use of endophyte associative diazotrophic bacteria as a biological input is a technology that may be utilized to supply nitrogen to crops. This technology has gained strength in the recent years as an alternative for sustainable production (Gosal et al., 2012; Sarathambal et al., 2015). These bacteria have the ability to promote plant growth by producing phytohormones as auxin, gibberellin and others, in addition to N_2 fixation to colonize the root tissue and shoots of plants (Fibach-Paldi et al, 2012; Kochar and Srivastava, 2012). Among the species of nitrogen fixing bacteria associated with forage grasses are *Azospirillum brasilense* and *Azospirillum lipoferum* (Tarrand et al., 1978), *Azospirillum amazonense* (Magalhães et al., 1983), *Azospirillum halopraeferens* (Reinhold et al., 1987), *Azospirillum doebereinerae* (Eckert et al., 2001) and *Azospirillum Milinis* (Peng et al., 2006).

The association between diazotrophic bacteria and several cultures such as grasses (forage plants), corn (*Zea mays*), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and sugarcane (*Saccharum officinarum* L.) has been of interest because of the biotechnology potential which is evidenced in the increase of productivity, reduces production costs, and maintains and conserves environmental resources (Moreira et al., 2010).This is the case of Brachiaria which has a high degree of association with *A. brasilense* species (Okumura et al., 2013). This association was observed by Hungria et al. (2016). These authors reported that there was a higher biomass production in plants inoculated with *Azospirillum brasilense* treatment combined with nitrogen fertilization of 40 kg N ha^{-1}.

Thus, the objective of this work was to evaluate the nutrition of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria grown in Cerrado soil (Brazilian Savanna).

MATERIALS AND METHODS

The experiment was conducted in a greenhouse at the Federal University of Mato Grosso, Campus Rondonópolis (16°28'15" S and 54°38'08" W).

The soil was classified as a Dystrophic Oxisol (Embrapa, 2011), collected at 0 to 0.20 m depth in an area under Cerrado vegetation (Brazilian savanna). Chemical and particle size analysis were conducted in accordance with the methodology proposed by Embrapa (2011). The soil presented the following chemical and physical characteristics: pH ($CaCl_2$ a 0.01 mol L^{-1}) = 4.6; organic matter = 18.8 g dm^{-3}; P = 1.4 mg dm^{-3}; K = 66 mg dm^{-3}; Ca = 1.1 $cmol_c$ dm^{-3}; Mg = 0.4 $cmol_c$ dm^{-3}; Al = 0.5 $cmol_c$ dm^{-3}; sand = 325 g kg^{-1}; clay = 525 g kg^{-1}; silt = 150 g kg^{-1}.

The base saturation of the soil was raised to 50% with the incorporation of dolomitic limestone with 80.3% effective neutralizing power (similar to TNRP – total neutralization relative power), reacting for a period of 30 days for the correction of soil acidity. During the limestone reaction in the soil, moisture was kept by the gravimetric moisture method at 60% of the maximum soil water retention, which was determined in the laboratory with pots with a corresponding volume to that used in the experiment, in three repetitions (Santos et al., 2013). After the limestone incubation period, fertilization was performed applying 200 mg dm^{-3} of phosphorus (using triple super phosphate as a source), 150 mg dm^{-3} potassium (using potassium chloride as a source) and 40 mg dm^{-3} of sulfur (using calcium sulfate as a source). The fertilization with micronutrients was made with 1.5 mg dm^{-3} of boric acid, 2.5 mg dm^{-3} of copper chloride, 2.0 mg dm^{-3} of zinc chloride and 0.25 mg dm^{-3} of sodium molybdate.

The experiment was conducted in a greenhouse in a completely randomized design with five treatments [(three strains of associative nitrogen fixing bacteria (MTH2 – *Bacillus* sp., MTB1- similar to *Burkholderia* sp. and Y2 – *A. amazonense*), an inoculant formed by combination of strains AbV5 and AbV6 (*A. brasilense*) and a control (100 mg dm^{-3} of N-urea)] in five replications. Each plot consisted of vases with 8 dm^3 capacity, totaling 25 experimental plots. Fifteen seeds (per plot) were sown directly into the pots. Seven days after sowing, the plants were trimmed, leaving five plants per pot.

The Y2 strain was cultivated in LGI medium (Döbereiner et al., 1995) and the other strains were cultivated in Dygs medium (Rodrigues Neto, 1986) under 100-rpm stirring, at 30°C, for 24 h. An aliquot containing 3 mL of bacterial broth (10^8 cells mL^{-1}) was applied to the soil around the root area of each plant. The appropriate number of cells was obtained using the most probable number method (Andrade and Hamakawa, 1994). Three cuts were made in the shoots at 30-days interval. The first cut was made 30 days after seedling emergence. The first and second cuttings were made at 5 cm from the base of the plant; the third cut was made close to the base of the plant, as described by Bonfim-Silva and Monteiro (2007).

Nitrogen and potassium fertilization was applied again after each cut in all experimental plots using the same doses and sources

Table 1. Treatment averages, F values and estimates of orthogonal contrasts of the Falker chlorophyll index for treatments within cuts of marandu grass submitted to inoculation with associative diazotrophic bacteria in Cerrado soil.

Treatment	Cuts		
	1º	2º	3º
	Average		
MTH2	40.16	21.14	24.48
MTB1	39.80	22.06	23.30
Y2	42.54	21.84	23.30
A. brasilense	39.64	20.40	22.22
Control	44.08	30.64	35.40
Contrasts	F_c		
C1	0.006**	0.000***	0.000***
C2	0.355ns	0.322ns	0.255ns
C3	0.460ns	0.553ns	0.389ns
C4	0.086ns	0.889ns	1.000ns
	Estimate		
C1	3.545	9.280	12.075
C2	-1.193	-1.280	-1.473
C3	-1.010	-0.810	1.180
C4	-2.740	0.220	0.000

C1 = Control vs. strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ns, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

shown above. 3 mL of broth corresponding to bacterial inoculation treatments, also inoculated with the same number of cells (10^8 cells mL^{-1}) was added. Chlorophyll index, the nitrogen concentration in shoots and roots, and crude protein in the shoots of marandu grass were analyzed. The chlorophyll index analysis was performed indirectly using a Falker chlorophyll meter clorofiLOG® model CFL1030. The shoot and root dry matter was performed using oven method. To determine the dry mass of shoot and root, the material was packaged in paper bags separately (shoots and roots) and placed to dry in an oven at 65°C for 72 h. Then, the dry matter values were obtained by weighing the material in a semi-analytical weight. The concentration of nitrogen and the crude protein was analyzed by the Kjeldahl method described by Malavolta et al. (1997).

Data were subjected to ANOVA, and the averages were analyzed by orthogonal contrasts and F-test using the SISVAR software (Ferreira, 2011). The contrasts applied to analyze treatments within each cut consisted of: (C1) = Nitrogen fertilization vs. strains of diazotrophic bacteria; (C2) = *Azospirillum brasilense* vs. other diazotrophic strains (MTH2, Y2 and MTB1); (C3) = MTH2 vs. Y2 and MTB1; (C4) = Y2 vs MTB1. The contrasts applied to analyze the cuts in each of the treatments consisted of: (C1) = First cut vs. second and third cuts; (C2) = Second cut vs. third cut.

RESULTS AND DISCUSSION

The mean values of orthogonal contrasts showed that there was a significant interaction between cuts and treatments for all the variables studied. In the deployment of the interaction of treatments within each cut, there was a significant effect for contrast 1 (C1) for all cuts. The first cut Y2 and MTH2 inoculation treatments showed mean values of 42.54 and 40.16 for the chlorophyll index. These values were close to the value observed in the control with nitrogenous fertilizer (44.08), which corresponds to the maximum production obtained. In other cuts, there were reductions in the chlorophyll index for all treatments (Table 1). The results observed for contrasts C1, C2 and C3, compared to nitrogen fertilization with inoculated treatments, indicate an influence of this treatment on the concentration of nitrogen in marandu grass leaves, allowing higher readings compared to the inoculated treatments. However, when the maximum values of chlorophyll index observed in marandu leaves that received nitrogen fertilizer was considered (44.08, 30.64 and 35.40), the Y2 strain with a value of 42.54 took 96.5% in the first cut. In the second and third cuts, the values reached 72.0% and 69.1% with the MTB1 and MTH2 strains respectively. It indicates that these strains have the potential to provide some of the nitrogen required for the development of marandu. Besides,the reading of the chlorophyll index is a parameter that can be used to evaluate the nutritional status of plants in relation to nitrogen (Argenta et al., 2001).

In the course of this study, when the chlorophyll content was assessed a better response in nitrogen fertilization was observed, this corroborates the observations reported by Abreu and Monteiro (1999) and Cabral et al. (2013), where the highest values of chlorophyll were observed in plants that received nitrogen fertilization. Viana et al. (2014) evaluated the effect of nitrogen fertilization on *Panicum maximum* cv. Tanzania grass and observed that this culture responded positively to the nitrogen doses applied. The values of chlorophyll content ranged from 35.53 to 47.85 at doses of 0 and 80 g dm^{-3} of nitrogen, respectively, and according to Maranhão et al. (2009), the chlorophyll content, in addition to indirectly measuring the nitrogen status of plants, may also be used as an indicator of the crude protein content of marandu grass. In the interaction of the cuts within each treatment, the highest values for chlorophyll index were observed in the first cut in all treatments. However, upon evaluating the other cuts, there was a significant effect for the third cut (Table 2).

The result observed for the first contrast (C1) indicates that marandu grass responded positively to the inoculation with associative diazotrophic bacteria, as well as to applied nitrogen doses, indicating the contribution of all nitrogen sources in the period related to the first cut in relation to others. Thus, the inoculation of associative diazotrophic bacteria may provide an increase in root surface, thus increasing the nitrogen that can be exploited in the soil and enabling the reduction of the use of nitrogen fertilizers (Okon and Vanderleyden, 1997; Gosal et al., 2012).

Table 2. Treatments averages, F values and estimates of orthogonal contrasts of the Falker chlorophyll index for cuts within treatments of marandu grass submitted to inoculation with associative diazotrophic bacteria in Cerrado soil.

Cut	Treatment				
	MTH2	**MTB1**	**Y2**	***A. brasilense***	**Control**
			Average		
1º	40.16	39.80	42.54	39.64	44.08
2º	21.14	22.06	21.84	20.40	30.64
3º	24.48	23.30	23.30	22.22	35.40
Contrasts			**Fc**		
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.037*	0.432ns	0.356ns	0.251ns	0.004**
			Estimate		
C1	17.350	17.120	19.970	18.330	11.060
C2	-3.340	-1.240	-1.460	-1.820	-4.760

C1: First cut vs. second and third cuts; C2: Second cut vs. third cut. ns, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

In contrast C2, the third cut in treatment with MTH2 strain was stressed, indicating that this strain was able to associate positively with marandu grass in comparison to other treatments with inoculation. In the third cut, the treatment with nitrogen fertilization was also stressed. It showed a positive response of this forage crop in relation to the application of mineral nitrogen. According to Olivares et al. (1997), the endophytic association allows these microorganisms to be free of competition with other groups of edaphic organisms present in the rhizosphere of plants, enabling a better use of the biologically fixed nitrogen.

The nitrogen-fixing bacteria provide this nutrient to the host plant, so that the greater its availability, the greater the amount of chlorophyll in leaves. Guimarães et al. (2011a) evaluated *Brachiaria decumbens* grass inoculated with *Azospirillum* spp. and observed a positive effect of these bacteria on the reading of the chlorophyll index in this grass, which was equivalent to 82% of the readings observed for the treatment with nitrogen fertilization (considered as maximum production), confirming that the association between the plant and bacteria may result in an adequate supply of nitrogen.

For Guimarães et al. (2011b), upon evaluating the effect of *Azospirillum* spp. inoculation on the productive characteristics of *Brachiaria brizantha* cv. marandu, there was a greater reading of the chlorophyll content in the treatment with nitrogen fertilization. However, among the inoculated treatments, the AZ18 strain had a value equivalent to 80% of that observed for plants that received N fertilization. This evidence shows that the inoculation with diazotrophic bacteria has the potential to partially replace nitrogen fertilization in low fertility soils (Itzigsohn et al., 2000).

In the outspread of treatments within the cuts for shoot nitrogen concentration, there was a significant effect in the treatment whose plants were inoculated with the MTB1 strain and in nitrogen fertilization in the third cut (Table 3). The nitrogen concentrations in this study were higher in the first cut compared to other cuts. This increase may be justified by the fact that young plants grow in relation to the expansion of leaf surface, where there are high concentrations of nitrogen that tend to decrease due to leaf senescence of forages over time (Muller et al., 2005).

Among the inoculation treatments with diazotrophic bacteria, the highest nitrogen concentrations in the first and second cuts were found in plant shoots inoculated with the MTB1 strain. This demonstrates that there is an association between the host plant and this strain when it is present in the rhizosphere of plants, because the rhizosphere of plants is considered a conditioner dynamics of microorganisms in the soil and can even interfere with the establishment of bacteria when inoculated in plants (Reis Junior et al., 2006).

In the third section, the concentration of nitrogen in the leaves of the inoculated treatments associate of diazotrophs showed an average of 47.3% of the maximum observed in plants corresponding to control with nitrogen fertilization (17.92). This shows that treatment with diazotrophs has economic value (compared to nitrogenous fertilizers), reduced negative effect on the environment and a contributing part of the nitrogen that is globally established by FBN (Reed et al., 2011)

Batista and Monteiro (2007), working with nitrogen levels from 1.0 to 33.0 mmol L^{-1}, observed that the influence of the concentration of this mineral in the blades of newly expanded leaves of marandu grass, ranges from 16.0 to 31.0 g kg^{-1} in a sample of the first cut and 9.5 to

Table 3. Treatment averages, F values, and estimates of orthogonal contrasts for the nitrogen concentration of the shoot of marandu grass in the outspread of treatments within each cut.

Treatment	Cut		
	1º	2º	3º
	Average (g kg^{-1})		
MTH2	22.68	7.2	8.12
MTB1	25.76	9.8	8.68
Y2	24.08	8.96	8.68
A. brasilense	19.04	3.36	5.88
Control	24.64	9.8	17.92
Contrasts		F_c	
C1	0.253^{ns}	0.108^{ns}	0.000***
C2	0.002**	0.001**	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
		Estimate	
C1	1.750	2.470	10.080
C2	-5.133	-5.293	-2.613
C3	-2.240	-2.180	-0.560
C4	1.680	0.840	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

17.0 g kg^{-1} in the second cut of the plants, and these results corroborates with the values found in this study, in both the first and second cut in all treatments observed for treatment with MTB1 and fertilized plants with nitrogen. The plants that received nitrogen fertilization had higher concentrations of this nutrient in the third cut, confirming the results obtained by Silva et al. (2005), according to which the nitrogen concentration in the shoots of marandu grass increased in function of nitrogen fertilizer doses. It is important to consider that even with the reduced concentration of nitrogen in the inoculation treatments in the second and third cuts, when the accumulation percentage of the inoculation treatments were compared with the maximum concentration observed in the control treatment, there were an approximation of 74.7 and 43.75%, respectively.

In all the cuts within each treatment, the first and second cuts had significant effects in the treatment with nitrogen fertilization, whereas for treatments with inoculation of diazotrophic bacteria, only the first cut had no significant effect (Table 4).

Costa et al. (2009), in studies with marandu grass subjected to doses and sources of nitrogen, observed that in the highest nitrogen doses, the concentrations of this nutrient in the plant were 18.86, 20.96 and 27.73 g kg^{-1} for the years 2004, 2005 and 2006, respectively, with an increasing linear effect over time. These results relate to this study, for they show that the nitrogen concentration tends to increase to the extent that this nutrient is available in a greater quantity to the plant. However, the nitrogen concentration decreased in the second cut in MTH2, *A. brasilense* and nitrogen control treatments increased the values in the third cut.

Cabral et al. (2013), in studies with marandu, convert and decumbens grasses, observed that for the first cut, in the absence of nitrogen fertilization, marandu and convert grasses had a higher concentration of nitrogen, inferring that these forages have a higher potential in the supply of nitrogen to ruminal microorganisms in the early stages of the forage grass development.

For the variable crude protein, the outspread of treatments within the cuts had a significant effect of diazotrophic bacteria strains on the first and second cuts. In the first cut, compared to nitrogen fertilization, the MTB1 strain showed an increase of 4.54%. The MTH2 and Y2 strains had crude protein values of about 92% and 97%, respectively, compared to the concentration of crude protein observed in the plants fertilized with mineral nitrogen (corresponding to the maximum production obtained). Higher concentrations of crude protein in the third cut were found in plants that received nitrogen fertilization (Table 5).

According to Minson (1990) and Van Soest (1994), pastures with levels below 7% of crude protein in dry matter are considered deficient for ruminants. Values below this level were observed in this study in the second cut for all treatments; in the third cut, it was satisfactory only for nitrogen fertilization. However, it corroborates the results obtained by Nicodemo et al. (2004), in which the authors found concentrations of crude protein ranging from 5.3 to 10.4% in pasture samples of *Brachiaria brizantha*.

Studying the crude protein content and the dry matter production of Brachiaria grass under nitrogen doses, Chagas and Botelho (2005) reported that the concentration of crude protein of this forage increased as the nitrogen rate increased, also in the analysis performed in the first cut.

In the outspread of cuts within each treatment, there were significant effects on the first cut in all treatments, and on the first and second cuts for nitrogen fertilization.

Similar to the results obtained in the nitrogen concentration in marandu leaves, there was a decrease in the second cut to MTH2, *A. brasilense* and nitrogen control treatments, increasing again at the third cut (Table 6).

In the first cut, treatment with MTB1 strain obtained a crude protein concentration similar to control, and only in the third cut the value of crude protein between these treatments was reduced. The lower production of crude protein observed in the inoculation in other treatments in the second and third cuts may have occurred due to a lower nitrogen accumulation in this phase, with a lower absorption of nitrogen and consequently a lower content of amino acids, proteins, nucleic acids, hormones and

Table 4. Treatment averages, F values, and estimates of orthogonal contrasts of nitrogen concentration in the shoot of marandu grass subjected to different cut and the interaction within each treatment.

Cut	Treatment				
	MTH2	MTB1	Y2	*A. brasilense*	Control
	Average (g kg^{-1})				
1º	22.68	25.76	24.08	19.04	24.64
2º	7.2	9.8	8.96	3.36	9.8
3º	8.12	8.68	8.68	5.88	17.92
Contrasts	F_c				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	15.020	16.520	15.260	14.420	10.780
C2	-0.920	1.120	0.280	-2.520	-8.120

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

Table 5. Treatment averages, F values and estimates of orthogonal contrasts of crude protein in the shoot in the outspread of interactions of treatments within each marandu grass cut submitted to inoculation with associative diazotrophic bacteria cultivated in Cerrado soil.

Treatment	Cut		
	1º	2º	3º
	Average (g kg^{-1})		
MTH2	14.17	4.50	5.07
MTB1	16.10	6.12	5.42
Y2	15.05	5.60	5.42
A. brasilense	11.90	2.10	3.67
Control	15.40	6.12	11.20
Contrasts	F_c		
C1	0.253^{ns}	0.108^{ns}	0.000***
C2	0.002**	0.001**	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
	Estimate		
C1	1.093	1.543	6.300
C2	-3.208	-3.308	-1.633
C3	-1.400	-1.362	-0.350
C4	1.050	0.525	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

Chlorophyll (França et al., 2007; Lavres Junior and Monteiro, 2003).

The decrease in crude protein observed in this study may be related to the fact that in tropical forage grasses, with advancing maturity, an early lignification of the tissues occurs and consequently there are changes in the cytoplasm of the plant with a decrease in protein and other nutrients due to the gradual increase of cell wall constituents (Coward-Lord, 1972). Pronounced decreases in crude protein content as the interval between cuts increases have been reported for various grasses under a tropical climate (Ruggieri et al., 1995). In the interaction of treatments within each cut for the concentration of nitrogen in roots, there was a significant effect of the inoculant in the first and second cuts, with a significant effect on the third cut for nitrogen fertilization (Table 7).

Nitrogen fertilization, as shown in the C1 contrast, was able to supply the nutritional requirement of marandu grass, providing the nitrogen needed for the crop during its development. For the treatments with diazotrophic bacteria, as seen in the C2 contrast, strains showed a substantial contribution to marandu grass nutrition, effectively providing nitrogen to the crop. The nitrogen accumulation in grasses because of a fasciculated root system has an advantage over the pivoting system of legumes to extract water and nutrients from the soil. Thus, even if part of the nitrogen is provided by the association with fixing bacteria, the economy of nitrogen fertilizer can be considered equal to that seen in legumes self-sufficient in nitrogen (Döbereiner, 1992).

Changes in the chemical composition of forage plants can be verified as maturation occurs. Thus, there is the decrease of components digestible by animals, increasing the fiber content and therefore decreasing the nutritional value of the feed (Cano et al., 2004). Batista

Table 6. Treatment averages, F values and estimates of orthogonal contrasts of crude protein in the shoot in the outspread of interactions of treatments of cuts within each marandu grass cut submitted to inoculation with associative diazotrophic bacteria cultivated in Cerrado soil.

Cut	Treatment				
	MTH2	**MTB1**	**Y2**	***A. brasilense***	**Control**
	Average (g kg^{-1})				
1º	14.17	16.10	15.05	11.90	15.40
2º	4.50	6.12	5.60	2.10	6.12
3º	5.07	5.42	5.42	3.67	11.20
Contrasts	**F_c**				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	9.387	10.325	9.537	9.012	6.737
C2	-0.575	0.700	0.175	-1.575	-5.075

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

Table 7. Treatment averages, F values and estimates of orthogonal contrasts for the nitrogen concentration in the roots of marandu grass by the outspread of interaction of treatments within each cut.

Treatment	Cut		
	1º	**2º**	**3º**
	Average (g kg^{-1})		
MTH2	2.26	0.72	0.81
MTB1	2.57	0.98	0.86
Y2	2.40	0.89	0.86
A. brasilense	1.90	0.33	0.58
Control	2.46	0.98	1.79
Contrasts	**F_c**		
C1	0.253^{ns}	0.108^{ns}	0.000***
C2	0.002**	0.001**	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
	Estimate		
C1	0.175	0.247	1.008
C2	-0.513	-0.529	-0.261
C3	-0.224	-0.218	-0.056
C4	0.168	0.084	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and ***- Not significant, significant at 1 and 0.1%, respectively, by F test.

and Monteiro (2006) demonstrated that the nitrogen concentration in the root tissue of marandu grass ranged from 29.5 g to 2.4 kg^{-1} in the nitrogen dose interval of 14-462 mg L^{-1} in the nutrient solution. In addition, in the study conducted by Maranhão et al. (2009) on the production and chemical composition of two Brachiaria cultivars fertilized with nitrogen, grass marandu showed a greater production of root, proving to be more resistant by

Table 8. Treatment averages, F values, and estimates of orthogonal contrasts for the nitrogen concentration in the roots of marandu grass by the outspread of interaction of cuts within each treatment.

Cut	Treatment				
	MTH2	**MTB1**	**Y2**	***A. brasilense***	**Control**
	Average (g kg^{-1})				
1º	2.26	2.57	2.40	1.90	2.46
2º	0.72	0.98	0.89	0.33	0.98
3º	0.81	0.86	0.86	0.58	1.79
Contrasts	F_c				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	1.502	1.652	1.526	1.442	1.078
C2	-0.092	0.112	0.028	-0.252	-0.812

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

exploring the soil more efficiently.

For the all the cuts within treatments, the nitrogen fertilization stood out in the first and second cuts, while the inoculation treatments presented a significant effect only in the first cut. Among the inoculation treatments, MTB1presented the highest nitrogen concentrations in the roots for the all cuts, exceeding the first cut and the values obtained in the roots of plants fertilized with mineral nitrogen (Table 8).

The nitrogen-fixing bacteria in association with plant roots, including forage, may assist in root growth and, therefore, plant growth by promoting substances such as phytohormones (Kuklinsky-Sobral et al., 2004; Pedraza, et al., 2004; Radwan et al., 2004). Thus, endophytic diazotrophic bacteria associated to plants may represent a promising alternative in promoting plant growth and soil management and, consequently, environmental quality.

With an area of 200 million hectares under pastures in Brazil, the appearance of areas in some stage of degradation, whether by soil management or use of fertilizer, especially nitrogen, is common. This may generate negative impacts on the environment (Boddey et al., 2006).

Thus, the use of diazotrophic bacteria associated to pastures aims to minimize the impacts caused by the indiscriminate use of nitrogen fertilizers, including contributing to sustainability in agriculture and livestock.

Conclusion

Associative diazotrophic bacteria contribute positively to the nitrogen nutrition of Marandu grass.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Serotyping and molecular typing of *Salmonella* species isolated from wastewater in Nsukka, Nigeria

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The objective of this investigation was to isolate and identify *Salmonella* serovars present in wastewater from the University of Nigeria, Nsukka (UNN) wastewater treatment plant and to evaluate the sensitivity and precision of different microbial typing methods (conventional and molecular), in identifying and characterizing *Salmonella* species. A total of 100 suspected *Salmonella* colonies on selective media (*Salmonella-Shigella* agar and MacConkey Agar) were subjected to biochemical testing. A total of 12 biochemically typical *Salmonella* isolates were identified and further characterized. Serotyping analysis further identified 3 (25%) of the isolates as *Salmonella enterica* serovar Limete. *Salmonella* specific (16S) polymerase chain reaction (PCR) assay validated the result obtained by serotyping, although 2 of the isolates could not be serotyped and were identified as rough strains. PCR assay produced positive amplifications of 574 bp of the 16S rRNA gene specific for *Salmonella*, while non-*Salmonella* serovars were negative (100%). Random amplified polymorphic DNA (RAPD-PCR) analysis revealed the genetic relatedness of *Salmonella* serovars isolated from wastewater. Primers 787 and RAPD2 identified 4 RAPD-binding patterns, while primer 1254 did not give any discriminatory pattern. Molecular analyses (16S PCR and RAPD) showed discriminatory power, reproducibility, easy interpretation and performance. It is therefore a promising alternative method for typing *Salmonella* species.

Key words: Wastewater, *Salmonella*, serovars, serotyping, polymerase chain reaction (PCR), 16S rRNA, random amplified polymorphic DNA.

INTRODUCTION

Wastewater may contain millions of bacteria per milliliter including coliforms, Streptococci, Staphylococci, anaerobic spore forming bacilli, *Proteus* and many other types of organisms. Wastewater is also a potential source of many human pathogens including bacteria, viruses and protozoa (Younis et al., 2003). The presence of microbial pathogens in polluted, untreated and treated water presents a considerable health risk to both humans and animals with far reaching socio-economic implications. *Salmonella* species have been frequently isolated from wastewater and are known to cause severe disease symptoms that range from self-limiting diarrhoea to bacteremia. They are the etiological agents of a wide range of diseases such as salmonellosis and typhoid

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fever and are among the leading causes of gastroenteritis worldwide. There are 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* (CDC, 2005; Bhunia, 2008).

Traditionally, detection and quantification of *Salmonella* species have been largely based on the use of selective culture and standard biochemical methods. This approach requires confirmatory test of all typical and atypical colonies and can be very cumbersome and time consuming (Whyte et al., 2002; Kumar et al., 2009). Since *Salmonella* is closely related to both human and animal health, more rapid and sensitive methods for the identification of this bacterium are required (Schrank et al., 2001). The use of DNA-based typing methods is becoming increasingly important in epidemiological survey and differentiation of *Salmonella* species. Some of these methods include pulsed field gel electrophoresis (PFGE) (Mohand et al., 1999; Kubota et al., 2005), PCR ribotyping (Lagatolla et al., 1996), automated nuclease PCR assay (Hoorfar et al., 2000) and random amplification of polymorphic DNA (RAPD) (Shangkuan and Lin, 1998; Smith et al., 2011). RAPD-PCR does not require any specific knowledge of the target DNA sequence, making it a flexible and powerful tool with general applicability. Furthermore, many oligonucleotide primer sets have been described for the detection of *Salmonella* using the PCR technique (Stone et al., 1994; Guo et al., 2000; Liebana et al., 2001).

In this study, the conventional system for typing *Salmomella* species (biotyping and serotyping) was evaluated and its performance was compared to molecular typing methods (16S and RAPD-PCR analysis).

MATERIALS AND METHODS

Sample collection

A total of 40 wastewater samples were collected from the Imhoff tanks (A and B) and waste stabilization ponds (WSPs) (A and B) of the wastewater treatment plant of the University of Nigeria, Nsukka (UNN), over a period of six months (October 2012 - March 2013). Wastewater samples were collected in sterile bottles and immediately transported to the Microbiology Laboratory, UNN and examined for *Salmonella*.

Isolation and identification of *Salmonella*

Salmonella species were isolated and identified according to the standard methods for the examination of water and wastewater described by ISO (2002) and APHA (2005). Forty milliliters (40 ml) of wastewater was centrifuged at 2000 rpm for 10 min, and then 10 ml of the supernatant was pre-enriched in 100 ml buffered peptone water (BPW) in a 250 ml Erlenmeyer flask and incubated at 37°C for 24 h. Pre-enrichment was followed by selective enrichment in Rappaport Vassilidiadis (RV) broth. Subsequently, selectively enriched samples from RV broth were streaked onto *Salmonella-Shigella* Agar (SSA) and MacConkey Agar. Plates were incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were subjected to a set of biochemical tests for confirmation.

Serotyping

All biochemically typical *Salmonella* isolates were serotyped based on reaction with somatic (O), flagellar (H) and capsular (Vi) antisera (Difco, USA). *Salmonella* O and Vi antigens were identified by slide agglutination test procedure. After the confirmation of the individual *Salmonella* O antisera, cultures were further characterized for H (phase I and II) antisera based on Spicer-Edwards antisera by tube test procedure, whereas, L, EN and I complex antigens were identified separately. The antigenic formulae of *Salmonella* serovars as listed by Popoff and Le Minor (2005) were used to name the serovars. Serotyping was carried out at the WHO Collaborating Centre for Reference and Research on *Salmonella* at Pasteur Institut, Paris, France.

Preparation of genomic DNA

Bacterial DNA was extracted by boiling according to the method described by Medici et al. (2003). A single colony of a pure nutrient agar culture was grown overnight at 37°C in 1 ml Luria Bertani broth. Bacterial cells were pelleted by centrifugation at 13,000 rpm for 5 min in a microcentrifuge (Eppendorf, Germany). The supernatant was discarded and the pellet was re-suspended in 500 µl deionized distilled water. The suspension was boiled for 10 min in a heat block (Techne, Barloworld, UK) then immediately cooled on ice. Extracted DNA was then stored at 4°C until used.

The extracted chromosomal DNA was amplified by an established PCR technique (Sambrook et al., 1989). Bacterial DNA amplification was carried out in a 25 µl total volume of PCR mixture containing 2 µl of template DNA, 4 µl of the PCR Master Mix (Solis BioDyne, Estonia) (1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1U Taq Polymerase), 20 pmol of each primer (Table 1), and ddH_2O to the total volume of 25µl. DNA amplification was done in an Eppendorf vapo protect thermocycler (Hamburg, Germany) following standardized cycling conditions (Table 2).

PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized with a photogel documentation system (Cjinx Science Instrument, USA). For RAPD-PCR, parameters in each reaction were optimized in order to maximize discriminatory power of the reaction. Also, for each PCR assay, a positive control (DNA from *S.* Typhi) and a negative control (sterile distilled water) were included. Appearance of the target band specified for each primer set under specified gel electrophoresis conditions was considered as a positive result.

RESULTS

Identification of *Salmonella* species from wastewater

The result showed that out of 100 *Salmonella*-like colonies on SSA and MacConkey agar, only 65 (65%) were found to be negative for urea utilization. Similarly, of the 65 urea negative isolates subjected to a set of biochemical reactions, 12 (18.4%) isolates were found to be consistent with results expected for *Salmonella* strains (result not shown).

Serotyping of *Salmonella* isolates from wastewater

Serotyping of *Salmonella* isolates from wastewater

Table 1. Primer sequence and reaction parameters.

Primer pair target	Primer sequence (5'–3')	Annealing temperature (°C)	No. of Cycles	Product size (bp)	References
16S rRNA gene	F: TGT TGT GGT TAA TAA CCG CA R: CAC AAA TCC ATC TCT GGA	56	35	574	Lin and Tsen (1996)
787	AAC GCG CAA C	36	30		Smith et al., 2006
RAPD2	CCC GTC AGC A	40	35		Smith et al., 2006
1254	CCG CAG CCA A	36	30		Smith et al., 2006

Table 2. Cycling conditions.

Step	RAPD primers			
	16sRNA	787	RAPD 2	1254
Initial denaturation	94°C/5 min	94°C/5 min	94°C/5 min	94°C/5 min
Final denaturation	94°C/1 min	94°C/5 min	94°C/1 min	94°C/1 min
Annealing	56°C/1 min	36°C/1 min	40°C/1 min	36°C/1 min
Initial extension	72°C/2 min	72°C/2 min	72°C/2 min	72°C/2 min
Final extension	72°C/10 min	72°C/5 min	72°C/7min	72°C/5 min
Hold	4°C	4°C	4°C	4°C

revealed that 3/12 (25%) isolates were identified as *Salmonella enterica* belonging to subspecies 1, serogroup B, named *S. enterica* serovar Limete (Table 3). The result further demonstrated that 2 of the isolates could not be serotyped and were identified as rough strains.

Number and percentage of *Salmonella* isolated from wastewater

The result demonstrated that 7.5% of wastewater tested was contaminated with *Salmonella* during the period of study. A total of 3 *Salmonella* isolates were detected and identified, 2 from Imhoff tank A and 1 from WSP B as shown in Table 4.

Salmonella specific PCR (16S rRNA) assay

The specificity and sensitivity of the 16S rRNA PCR assay revealed that 3/12 (25%) of the standard microbiological tests confirmed isolates from wastewater had the desired amplification of 574 bp fragment, whereas no amplicons were observed for non-*Salmonella* serovars (Figure 1).

RAPD-PCR of *Salmonella* isolates from wastewater

The discriminatory power of the RAPD-PCR assay was tested by considering the number of profiles (RAPD binding patterns) generated using a set of 3 primers. The result of the RAPD-PCR revealed that primers 787 produced 3 similar bands and RAPD 2 produced 1 uniform band for the 3 isolates (Figure 2), making a total of 4 RAPD binding patterns on the basis of genetic relatedness. Primer 1254 did not produce any discriminatory pattern amongst the *Salmonella* isolates.

DISCUSSION

The result of this study revealed a low occurrence (7.5%) of *Salmonella* spp. in wastewater collected from the University of Nigeria, Nsukka wastewater treatment plant (WWTP). This is in consonance with the work done by El Hussein et al. (2012) in Khartoum State, Sudan, who reported an

Table 3. Serotypes of *Salmonella* isolates from wastewater.

Sample code	Detected O-antigen	Detected H-antigen	Complete antigenic identity (O and H)	Serotype identification (*S. enterica*)
A1	-	-	-	-
A5	O:4, O:12[27]	b:1, 5	1, 4, 12, [27]: b.1, 5	*S.* Limete
A6	O:4, O:12[27]	b:1, 5	1, 4, 12, [27]: b.1, 5	*S.* Limete
A12	-	-	-	-
A17	-	-	-	-
B18	-	-	-	-
B24	-	-	-	-
B28	-	-	-	-
C1	-	-	-	-
C8	O:4, O:12[27]	b:1, 5	1, 4, 12, [27]: b.1, 5	*S.* Limete
C28	-	-	-	-
D11	-	-	-	-

A = Samples from Imhoff tank A; B = Samples from Imhoff tank B; C = Samples from Waste stabilization pond A; D = Samples from waste stabilization pond B.

Table 4. Number and percentage of *Salmonella* isolated from the wastewater treatment plant, UNN.

Source	Total samples examined	Total positive samples	Source (%)	Positive samples (%)	Total examined (%)
Imhoff Tank A (inlet)	10	2	20	66.7	5
Imhoff Tank B (outlet)	10	0	0	0	0
WSP A	10	1	10	33.3	2.5
WSP B	10	0	0	0	0
Total	40	3	-	100	7.5

WSP = Waste stabilization pond.

Figure 1. *Salmonella*-specific PCR (16S rRNA) of isolates from wastewater. Lane M: 100 bp DNA, Lane 1: Negative control, Lane 2: Positive control (*S.* Typhi), Lane 3-14: samples, Lane 9, 10 and 13 reveals amplification of 574 bp fragments of 16S rRNA gene.

occurrence of 11.09% of *Salmonella* species in wastewater. Conversely, a report by Howard et al. (2004) showed that municipal wastewater having undergone an activated sludge process continued to bear *Salmonella* at

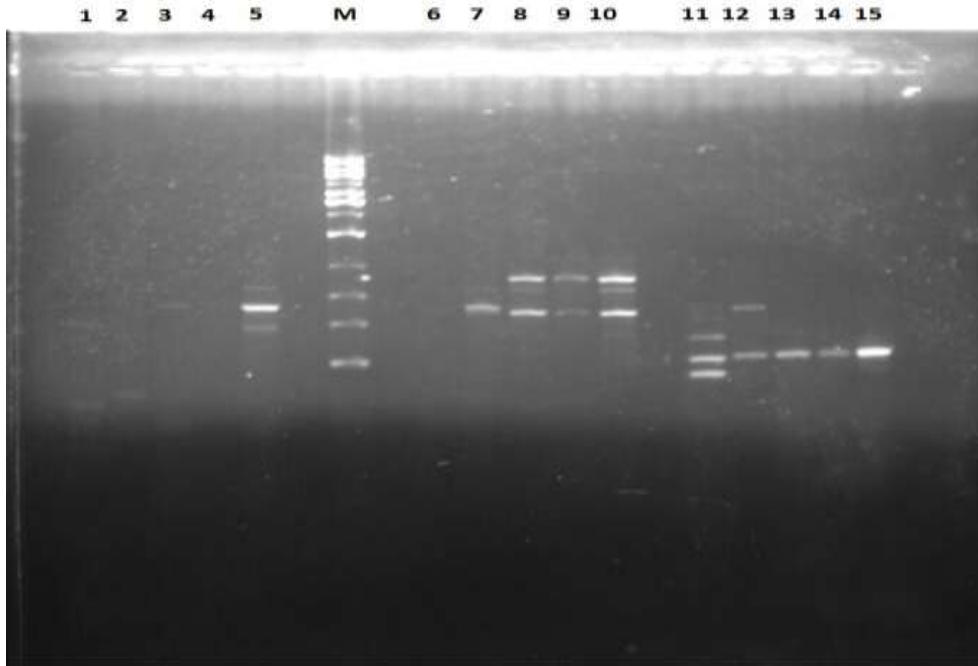

Figure 2. Representative RAPD-PCR of *Salmonella* isolates from wastewater using a set of 3 primers (1254, 787, RAPD 2). Lane M: 1 kb Marker, Lane 3 - 5 (1254), Lane 8 - 10 (787), Lane 13 - 15 (RAPD 2).

high concentration; the treated water yielded a most probable number (MPN) of 45/100 ml. Also, El-Taweel (1994) found that *Salmonella* spp. were detected in raw wastewater samples at oxidation pond in Mit-Mzah treatment plant in Dakahlia governorate, Egypt, in numbers ranging from 10^2 - 10^5 cfu/100 ml.

The result of serotyping was in tandem with the result of the 16S rRNA-PCR assay, although 2 isolates could not be serotyped and were identified as rough strains. Kumar et al. (2009) reported that some of the *Salmonella* serovars isolated from seafood could not be serotyped and were identified as rough strains, lacking O-antigen. This reveals a major limitation of the serotyping technique in subtyping *Salmonella* spp.; as serogrouping is not possible when *Salmonella* isolates lack O-antigen (rough strain) or lack both O and H antigens (Hoorfar et al., 1999). This highlighted the necessity of more discriminatory methods to compliment traditional typing methods. However, since the PCR method could not also identify these as Salmonella, it is inferred that this may not have been a limitation in this study.

The specificity and sensitivity of the PCR assay to detect *Salmonella* spp. recovered from wastewater was investigated. The PCR produced positive amplifications of 574 bp of the 16S rRNA gene specific for *Salmonella* serovars, while non-*Salmonella* serovars were negative (100%). The results obtained were similar to those reported by Lin and Tsen (1996) and Chiu and Ou (1996). These investigators reported that 16S PCR technique using Salm 16S primer was able to identify all the examined *Salmonella* serovars, while all non-*Salmonella* serovars gave negative results.

There was no diversity among the strains analyzed by RAPD-PCR. The discriminatory power of the RAPD-PCR was tested by considering the number of profiles (RAPD binding patterns) generated using a set of 3 primers. The analysis of the RAPD-PCR revealed that primers 787 and RAPD 2 were found useful in typing *Salmonella* isolates and 4 RAPD patterns were observed among the isolates on the basis of shared amplified product showing relatedness of the isolates. Primer 1254 did not produce any discriminatory pattern amongst the *Salmonella* isolates and obviously no typing was possible. This result is in consonance with studies conducted in Lagos, Nigeria by Smith et al. (2011) and Akinyemi et al. (2014). These investigators demonstrated that RAPD-PCR using primer 1254 did not discriminate among *Salmonella* isolates. However, this is in contrast with the report of Quintanes et al. (2004) which recorded highest discriminatory power amongst clinical *Salmonella* isolates using Primers 784 and 1254 in Brazil.

Standardization of PCR mixtures and conditions are very important for reproducibility of RAPD-PCR results. It was found that it was necessary to perform RAPD-PCR in duplicates to obtain valid result. It is important to note that the interpretation of DNA fragment patterns generated by RAPD-PCR requires a good understanding of the occurrence of random genetic events, including

point mutations, insertion and deletions of DNA, which can alter the RAPD fingerprinting pattern (Tenover et al., 1997). For this reason, it was presumed that the differentiating bands in the profiles could be due to one or more genetic events. These findings show that RAPD-PCR yields reliable and reproducible results under precise assay conditions.

The traditional phenotypic typing methods used (biotyping) showed low discriminatory power, while serotyping yielded inconclusive results in few samples. RAPD-PCR analysis showed the potential to provide a discriminatory, reproducible, low cost, easy to perform and interpret method to type *Salmonella* strains. Molecular typing or fingerprinting is an invaluable epidemiological tool that can be used to track the source of infection and to determine the epidemiological link between isolates from different sources. The combination of traditional and molecular typing methods may be the best approach to characterize *Salmonella* isolates.

Conflict of Interests

The authors have not declared any conflict of interests.

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Reduction of organic contaminants and microbial communities in bioecological wastewater treatment system

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Wastewater treatment is a key factor in controlling eutrophication of freshwater bodies. Nutrients discharge in wastewater can cause water quality problems such as eutrophication of freshwater bodies, decreased conservation and recreational value of water systems, and destruction of aquatic life. A pilot scale bioecological wastewater treatment system which consisted of a modified anaerobic\anoxic\oxic (A_2O) system as a biological part and constructed wetland as an ecological part was developed to treat domestic wastewater (sewage). The study was carried out for pollutant removal performance of system and identification of microbial communities present in the system. The system showed excellent removal efficiency for chemical oxygen demand (COD) and organic nutrients such as ammonia, total nitrogen (TN) and total phosphorus (TP). Microbial count and distribution in different units of the bioecological system were dependent on associated factors such as, oxygen level, nutrients concentration and filtration from substrate. The bioecological system was proved to be quite effective in reducing total bacterial count (60%) as well as fecal coliform. The system offers simple operation, low energy consumption and high removal efficiency.

Key words: Wastewater, eutrophication, bioecological, constructed wetland, water quality.

INTRODUCTION

Raw wastewater contains biodegradable organic and inorganic compounds, toxic substances and microbial pathogens. The discharge of untreated wastewater is unsafe, both from health and environmental perspectives (Sehar et al., 2013). For the past several years, wastewater treatment technologies have improved mainly due to the more stringent nutrient discharge limits. Currently, wastewater treatment has become one of the world largest technologies for environmental protection due to increase in industrialization, urbanization and population growth (Nielsen et al., 2010). The practice of wastewater treatment technology is based on several

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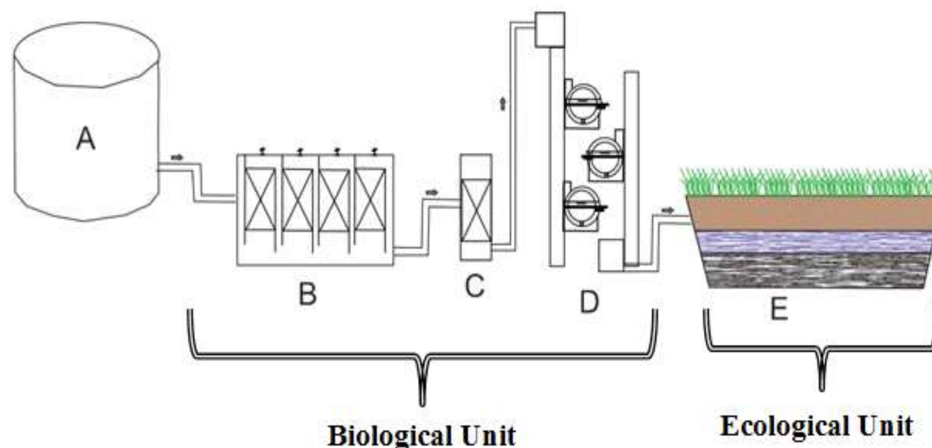

Figure 1. Schematic diagram of the bioecological system; A. Wastewater storage tank; B. Anaerobic baffled reactor; C. anoxic tank; D. Oxic unit (the unit consisted of three sub-units, each unit consisted of a rectangular tank, a rotating disc and a bio-wheel rotating disc); 7. Horizontal flow constructed wetland indicating the directions of wastewater flow

factors, such as origin and composition of wastewater, availability of land, skilled persons, population settlement in the community, etc. (Jhansi and Mishra, 2013). The conventional centralized wastewater treatment technologies are not suitable for the treatment of small populations and wastewater of rural areas because of the scattered and isolated locations which make collection of wastewater difficult; furthermore, high variability in flow rate and nutrients load is also an issue (Zhang et al., 2009). The sustainability of planet earth and reuse of its limited resources is a primary concern. Urban infrastructure and conventional treatment systems are built based on cost, convenience, the technology available and discharge limits for the treated wastewater (Abbasi et al., 2016).

Bioecological wastewater treatment is the combination of biological system and ecological system. For the past several years, anaerobic-anoxic-oxic (A_2O) system and constructed wetland (CW) has been applied to treat a different type of wastewater (Jin et al., 2014). In a bioecological system, various types of microorganisms play a significant role in nutrient removals such as nitrifying and denitrifying bacteria for nitrogen removal and phosphate-accumulating organisms (PAOs) for phosphorus removal (Kim et al., 2013). The function of the bioecological system has been characterized by raw wastewater compositions (chemical oxygen demand, total suspended solids and nutrients) and operational conditions, such as hydraulic retention time (HRT), pH, temperature and dissolved oxygen (DO) (Chan et al., 2009).

Many studies have shown that the impacts of global warming and climatic changes influence the temporal and spatial distribution of precipitation and hydrological cycles subsequent changes in water bodies (Zhang et al., 2010). The bioecological system is an alternative approach to avoid the disadvantages of conventional wastewater systems. Wastewater and its containing nutrients are recognized as a resource (not waste), which should be made available for reuse (Langergraber and Muellegger, 2005). The studied bioecological technology possesses biological unit and an ecological unit. The biological unit is A_2O (anaerobic/anoxic/oxic) and the ecological unit is CW (constructed wetland). The aim of this research was to study the performance of the bioecological system concerning chemical and biological pollutants and identification of microbial flora in different units of the system. The system requires simple construction and is easy for operation and maintenance with little cost. Recovering nutrients, reusing treated wastewater and consumption of low energy make bioecological wastewater treatment more sustainable (Abdel-Raouf et al., 2012).

MATERIALS AND METHODS

Reactor setup

A lab-scale bioecological wastewater treatment system consisted of two parts, a biological unit and an ecological unit (Figure 1).

Biological unit

Biological unit is A_2O system; consisted of an anaerobic baffled reactor (ABR), anoxic tank and oxic part. The anaerobic baffled reactor (ABR) was 1 m long, 0.2 m wide and 0.75 m high with 100 L effective volume. The reactor had five compartments divided by vertical baffles and filled with non-woven cloth. The anoxic tank was 1 m high, 0.2 m long and 0.2 m wide with 32 L effective volume and equipped with outlets at different heights. The oxic unit consisted of three aerobic turntable cells and each cell consisted of a rectangular tank (0.2 × 0.2 × 0.1 m in diameter), a rotating disc and a bio-wheel rotating disc, working on the watermill principle.

Ecological unit

The ecological unit was a horizontal flow constructed wetland (CW) consisting of a rectangular polyvinyl chloride (PVC) container (Length × width × height = 1× 0.2 × 0.6 m). The ecological unit was packed with three layers one over the other (gravel 15 cm as supporting layer, cobblestone (Yao et al., 2013) cm, sand and soil mixture 5 cm). The unit was planted with *Apium graveolens* (celery) because of its economic value in the local market. Horizontal subsurface flow constructed wetlands are widely used for treating domestic wastewater (Vymazal, 1996).

Experimental procedure

The experimental reactor had been running for over four months after system start-up. In the biological unit, ABR startup is a complicated process and need time to maintain full treatment capacity (Yu and Lu, 2014). ABR startup can be affected by many factors such as wastewater concentration and composition, pH, hydraulic retention time (HRT), temperature and reactor size (Hassan et al., 2015). For a start-up, the ABR operated for 50 days with different HRTs. Initial HRT was 72 h for 20 days and gradually reduced to 48 h for 15 days and later 24 h until the COD removal efficiency stabilized at 60%, and the pH stabilized between 7.03 and 7.23. The temperature during the whole study period was 20-32°C. The biological unit directly received wastewater from the storage tank, and after treatment from the biological unit, the effluent water was pumped to the ecological unit. Valves, nozzles and pumps were used to regulate the flow rate of water from one unit to another.

Sewage characteristics

The raw sewage for this study was obtained from the campus of the Southeast University at Wuxi. The average pH of raw sewage was 7.06, chemical oxygen demand (COD) 258.4 mg/L, TN 33.8 mg/L, NH_4^+-N 25.6 mg/L, TP 4.3 mg/L and TSS 276 mg/L. The wastewater was generated from dormitories, laboratories and restaurants on the University Campus.

Analytical methods

Chemical oxygen demand (COD), ammonia (NH_4^+-N), total nitrogen (TN), total phosphorus (TP), and total suspended solids (TSS) were analyzed according to standard methods (Federation and Association, 2005). Dissolved oxygen (DO) and pH were analyzed by DO200 and PH100 probes (YSI), respectively.

Bacterial profiling and microbiological analysis

Bacterial diversity colonizing and microbiological analysis in the bioecological unit were studied. For this purpose, 50 ml of the water sample from the effluent of each part of the bioecological unit was collected and serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) were prepared in phosphate buffer saline (PBS) by serial dilution method (Holt, 1994). The appropriate dilution was selected and 1 ml water was taken by pipette and poured on nutrient agar (NA) plates. The water was spread on NA agar plates by using a sterile spreader and the plates were incubated at 37°C for 24 h. After incubation, colonies were distinguished by morphology, size and color.

For bacterial profiling and to obtain pure cultures, different colonies were further sub-cultured on *Salmonella-Shigella* agar (SSA), eosinmethylene blue agar (EMB), *Pseudomonas* cetrimide agar (PCA), mannitol salt agar (MSA), MacConkey's agar (MacA) and blood agar (BA). These plates were again incubated for 24 h at 37°C. On the basis of morphology, microscopy and biochemical characteristics, sub-cultured organisms were identified.

A microbial analysis was carried out by most probable number technique (MPN index) and colony forming unit (CFU/mL). The number of colonies was counted by placing the NA plate under colony counter and calculation was made according to the following formula:

CFU/mL = number of colonies × dilution factor/inoculum size

For the investigation of coliform, fecal coliforms and pathogens (*Escherichia coli*, *Shigella, Salmonella*, Klebsiella sp., *Citrobacter* and *Enterobacter*) samples were incubated in MacConkey's broth for 24-48 h at 42.2°C. Positive tubes were sub-cultured on NA, MacAand MSA plates and incubated for 24-48 h at 37 ± 2°C. Gram staining and light microscopy were done for the positive cultures to differentiate the Gram positive and Gram negative microorganisms.

Data analysis

SPSS version-18.0 (SPSS incorporation Chicago, Illinois, USA) and MS-excel programs were used for data analysis and presentation.

RESULTS AND DISCUSSION

Bioecological wastewater treatment system holds lots of attraction since is based on natural cycles instead of chemical and mechanical systems to treat wastewater (de-Bashan and Bashan, 2004).

Removal of organic pollutants and nutrients

Organic material and nutrients are crucial components of municipal wastewater which are responsible for eutrophication (Shon et al., 2006). The average influent concentration, effluent concentration and removal efficiencies of COD, ammonia, TN and TP during the study period are shown in Table 1. Influent concentrations of pollutants were between 180 to 321, 22 to 42, 17 to 36 and 3.2 to 5.5 mg/l for COD, TN, ammonia and TP, respectively. The variation in the influent concentration is because of the instable and discontinuous quantity and quality of the sewage water entering the system and impact of rainwater that diluted the raw sewage. The average effluent concentrations was 19.6 ± 4.6, 7.96 ± 1.84, 2.35 ± 0.99 and 0.36 ± 0.10 mg/l with the removal efficiency of 92, 78, 92 and 92% for COD, TN, ammonia and TP, respectively. The bioecological system showed highly significant effect ($P < 0.001$) for COD, TN, ammonia and TP.

Figure 2 shows the removal rate of pollutants in different units of the bioecological system. The system had an anaerobic, anoxic and oxic phase with a CW. The most effective COD removal took place in the anaerobic unit and it was supposed that the heterotrophic bacteria were responsible for chief quantity of organic matter removal (Yao et al., 2013). The average removal rate of

Table 1. Statistical analysis of pollutants.

	Influent (mg/L)			Effluent (mg/L)			Efficiency (%)		
	Min	Max	Mean ± sd**	Min	Max	Mean ± sd**	Min	Max	Mean
COD	185	321	268.2 ± 30.75	10	28	19.6 ± 4.6	90	95	92
TN	22	42	36.01 ± 4.59	3.6	11.2	7.96 ± 1.84	71	84	78
Ammonia	17	36	29.9 ± 4.42	0.5	4.6	2.35 ± 0.99	86	97	92
TP	3.2	5.7	4.7 ± 0.59	0.2	0.58	0.36 ± 0.10	87	95	92

COD stand for chemical oxygen demand, whereas TN is total nitrogen and TP represents total phosphorus. **is the probability value > 0.001.

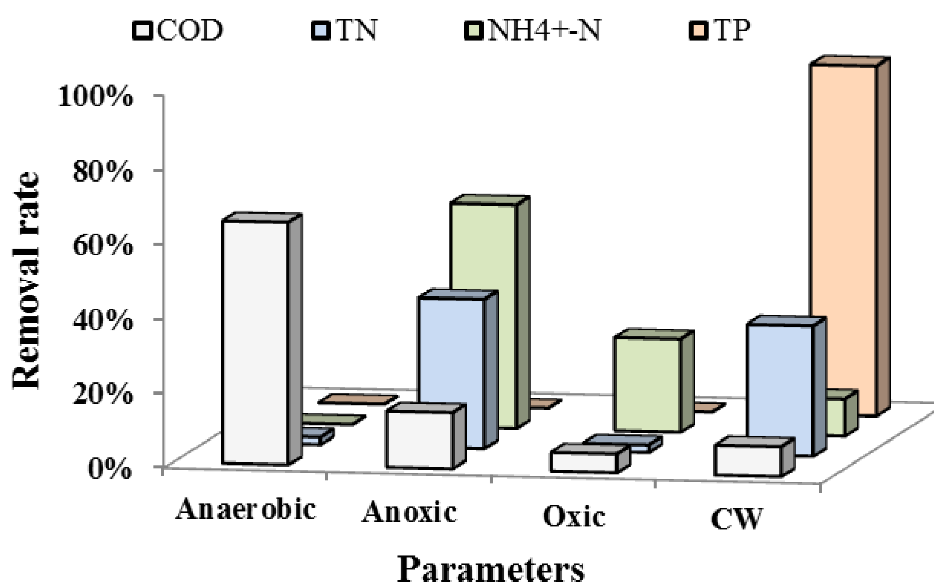

Figure 2. Removal efficiency of pollutants in different units of bioecological system.

TN in the anoxic tank and CW was 40 and 35%. Nitrogen removal occurred through nitrification, denitrification, volatilization and by plant uptake (Ye and Li, 2009). One of the primary functions of CW is nitrogen uptake by plants (Tunçsiper, 2009). The main part of TP was removed by the CW and adsorption, biological oxidation and plant uptake were the main processes for TP removal (Mina et al., 2011).

MPN Index of wastewater

Human excreta in municipal wastewater contain pathogenic organisms and consequently may be hazardous. Figure 3a and b show the variation of colony-forming unit (CFU) and MPN index different units of the bioecological system. Microbiological existence, survival and distribution are affected by the type of wastewater treatment units and associated factors (Cabral, 2010). The bacterial count for raw sewage was 9.3×10^{7} CFU/mL. It shows an increasing trend of microbial count in ABR compartment I, II and III. This was possibly the result of elevated substrate concentration. Therefore, presence of conditions and nutrients for anaerobic microbial growth resulted in increased microbial number (Movahedyan et al., 2007). The compartment IV and V show low number of CFU and MPN, possibly because the less number of available nutrients for anaerobic microbial growth, as most of the nutrients, were consumed in first three compartments. In the whole bioecological system, the oxic unit shows highest number of bacterial count. Similar observations were found by Kim et al. (2013) for A_2O process and obtain 1,546, 2,158 and 3,743 reads in anaerobic, anoxic and oxic chambers, respectively. Although, there were increasing trend in bacterial count from ABR to oxic unit because of functionality and atmospheric conditions (Liu et al., 2007), however effluent from the final stage of bioecological system which was CW shows the lowest number of bacterial count. The sand bed present in CW successfully decreases bacterial count in wastewater after treatment from the bioecological system (Guchi, 2015).

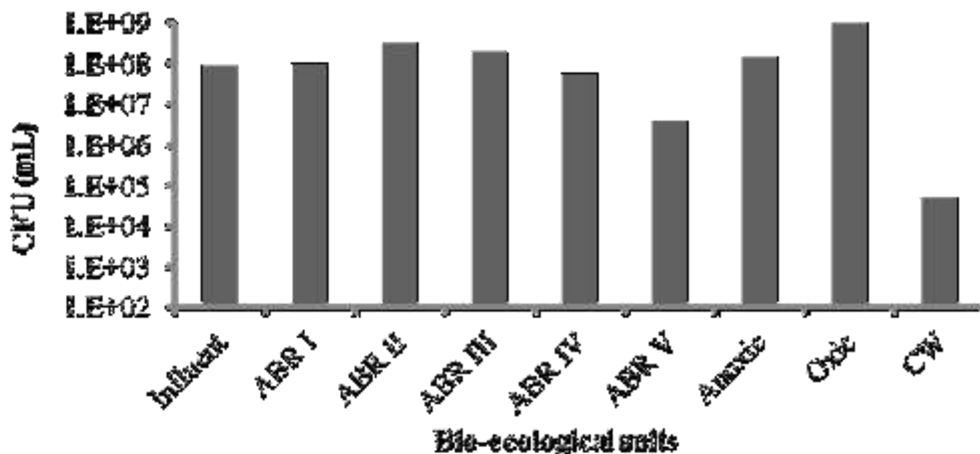

Figure 3a. Estimation of colony-forming unit (CFU) in different units of bioecological system.

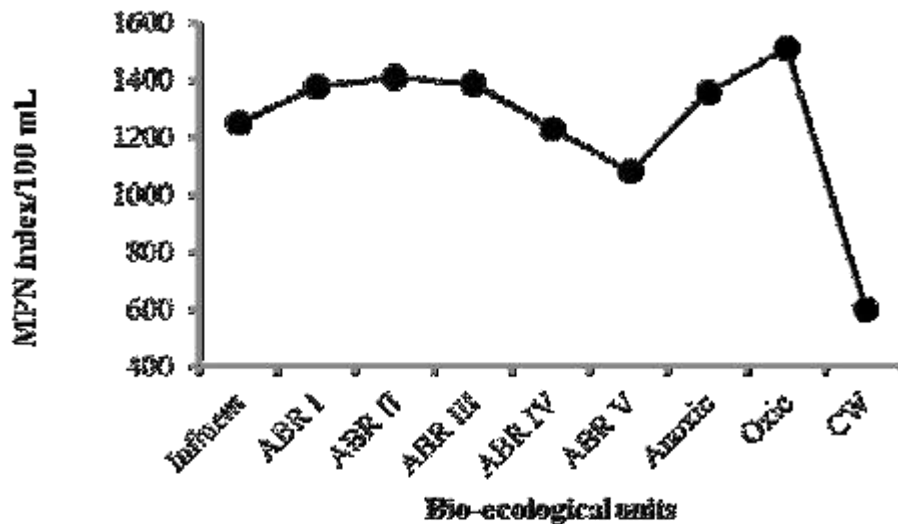

Figure 3b. Estimation of most probable number (MPN) in different units of bioecological system.

Bacterial profiling

The faces of a single individual may contain at least 300 different species of bacteria. Most of these bacterial species are strict anaerobes and remaining facultative anaerobes. *E. coli* is a common facultative anaerobe in feces. Bacteria from human excreta and other sources enter into the influent wastewater treatment system. Bacterial profiling of bioecological wastewater treatment system was performed in different units independently. The bioecological system serves as an important reservoir for accumulation of various types of microorganism groups. Morphological, microscopic and biochemical analysis were performed to isolate and identify the bacterial strains by following the protocols of Bergey's Manual of Determinative Bacteriology (Vos et al., 2011) from the water samples of bioecological systems (Table 2).

According to these findings influent sample was positive for *Staphylococcus aureus*, *Proteus* species, *Klebsiella spesies, Alcaligenes faecalis, Salmonella* species, *Escherichia coli, Shigella* species, *Bacillus* species, *Pseudomonas* species, *Enterobacter* species and *Micrococcus* species. In ABR, *Enterobacter, Shigella, Pseudomonas, Proteus, Shigella, Klebsiella* and *E. coli* species were positive in all compartments of ABR (ABR I, II, III, IV and V), whereas *Staphylococcus, Micrococcus, Bacillus* and *Alcaligenes* species was present only in ABR I. The presence of aerobic microflora in ABR I was found because it was positioned just next to

Table 2. Diversity of bacterial species in different units of bioecological system.

Species	Influent	ABR					Anoxic	Oxic	CW
		I	II	III	IV	V			
Staphylococcus aureus	+	+	-	-	-	-	+	+	+
Micrococcus species	+	+	-	-	-	-	-	+	+
Bacillus species	+	+	-	-	-	-	-	+	-
Enterobacter species	+	+	+	+	+	+	-	-	-
Shigella species	+	+	+	+	+	+	-	-	+
Pseudomonas species	+	+	+	+	+	+	+	+	+
Proteus species	+	+	+	+	+	+	+	+	+
Shigella species	+	+	+	+	+	+	+	-	+
Klebsiella species	+	+	+	+	+	+	-	-	-
Escherichia coli	+	+	+	+	+	+	+	+	-
Alcaligenes faecalis	+	+	-	-	-	-	-	+	-

storage tank, as the anaerobic conditions linger in other compartments of ABR (II, III, IV and V) the aerobic bacteria were unable to survive in absence of molecular O_2 (Kato et al., 1997).

The anoxic tank was positive for *S. aureus, Proteus* species*, Pseudomonas* species*, Klebsiella* species and *Escherichia coli. Bacillus* species*, Micrococcus* species, *Staphylococcus aureus, Pseudomonas* species, *Proteus* species, *Escherichia coli* and *Alcaligenes* species were isolated from the oxic unit. Whereas, the water sample of CW was positive for *Pseudomonas*, *Shigella*, *Proteus*, *Salmonella, S. aureus* and *Micrococcus* species.

Conclusion

Based on the conducted study, the main findings are as followings: The bioecological system performed very well for COD, TN, ammonia and TP removal: 92, 96.7, 83.6 and 95.3% removal efficiency, respectively. Microbial count, distribution and survival in different units were dependent on associated factors such as, oxygen level, nutrients concentration and filtration from substrate. The bioecological system was proved to be quite effective in reducing bacterial count as well as fecal coliform. The system is a low cost, energy saving, an alternative and appropriate technology to wastewater treatment, in particular for the rural regions.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Reconstructed H3N2 influenza virus predicted from influenza vaccine strains improved cross-protective immunity in mice

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Hemagglutinin (HA) protein of influenza virus is a core antigen protein which induces protective antibody in hosts. But HA genes mutate rapidly. Every year, World Health Organization (WHO) selects representative influenza virus strains from the influenza centers worldwide for virus vaccine production. So, the selected influenza vaccine strains, can partly respond to antigen drifts of circulating influenza virus, especially the reconstructed H3N2 which induces the cross reaction. It is reported here using the Immune Epitope Database and the reverse genetic method on how to produce reassortant influenza virus based on the changes of B and D antigenic regions (B antigenic region: 156-160aa,187-198aa; D antigenic region:167-182aa, 201-215aa) of HA protein of seasonal influenza H3N2 vaccine strains over twenty years. In a mouse model, the attenuated reassortant viruses induced neutralization antibodies, cross-reactive T-cell responses, and were protective against different lethal influenza virus challenge. So, through the analysis of the antigenic regions of HA using computer and software methods, the reconstructed rB/D influenza virus mostly induced cross protection in mouse model.

Key words: influenza, vaccine strains, antigen epitope, cross-protective.

INTRODUCTION

Influenza virus causes influenza epidemics, which continues to impose a significant impact on the world's population, especially resulting in human suffering and economic burden (Nair et al., 2011; Molinari et al., 2007). Currently, the major subtypes circulating in human populations are influenza A/H3N2 and A/H1N1 subtypes (Pediatrics, 2012; Harper et al., 2009; Rambaut et al., 2008). HA is A special factor due to its role in the viral entry mechanism and immune recognition (Ge et al., 2010; Babon et al., 2012; Bean et al., 1992). It consists of two subunits, HA1 and HA2. Through continuous antigenic mutations, HA1 contains the receptor-binding

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and antigenic domains, experience a process termed positive Darwinian selection (Corti et al., 2010; Han and Marasco, 2011). The variable antigenic regions of the HA1 domain are potential targets of neutralizing antibodies. And thus, amino acids substitutions at these regions (A to E) have been associated with annual epidemics in humans (Carrat and Flahault, 2007; Wang et al., 2009; Suzuki, 2008). The influenza vaccines are used for the prevention of influenza (Centers for Disease Control and Prevention, 2013; Sook-San et al., 2013). The strain that will cause the pandemic could not be predicted (Kaminski and Lee, 2011; Wright, 2008). Therefore, development of a vaccine that induces broadly cross-protective immunity against variant viruses is urgently needed (Gomez et al., 2013).

In this study, it was discovered that some amino acids changes of B/D antigenic regions were critically important in improving IgG and IgA antibodies and T cell immunity. In addition, results showed that reassorant virus induced cross-protective immunity in mice. It was predicted that HA1 antigenic regions change in seasonal H3N2 influenza vaccine strains during the past twenty years. It was discovered that amino acid substitutions occurred mainly in B and D antigenic regions of HA protein. Through the prediction of T cell's epitopes, the relationship between amino acids changes of B and D antigenic regions and its immunogenicity and heterologous protection were investigated.

MATERIALS AND METHODS

Influenza vaccine strain (H3N2) HA amino acid sequences

The major surface analyzed was glycoprotein hemagglutinin (HA). The Influenza Research Database website and NIBSC (National Institute for Biological Standards and Control) was used, and the downloaded seasonal influenza A vaccine strains gene pregments which had whole full-length HA protein in the past twenty years (World Health Organization 2015).

Sequence analysis

Bio Edit version 7.0.9.0 was used for HA protein analysis. The four antigenic regions: A, B, C and D were compared in H3 HA molecules (Figure 2). HA proteins were compared to predict the conserved T cell epitopes. The Immune Epitope Database (IEDB) was used to predict CD4+ and CD8+ T cell epitopes of HA proteins. Human leukocyte antigen (HLA)-DRB1 alleles were selected to identify the CD4+ T cell epitopes, Netmhcllpan (3.1 Server) was selected as prediction method. The prediction values are given in nM IC50 values and as % Rank to set of 200 random natural peptides. Threshold for strong binding peptides (IC50): 50.000 nM; Threshold for weak binding peptides (IC50): 500.000 nM; Affinity (nM) were predicted with binding affinity in nanomolar IC50.

Virus cell culture

Madin-Darby canine kidney (MDCK) (ATCC: CCL-34) and 293T human embryonic kidney cell (ATCC) were grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing (1%) bovine serum albumin (BSA). DMEM/F12 (pH 7.0-7.2) containing (1%) bovine serum albumin (BSA), 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES buffer and (1%) L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin were used for virus growth. A/TaiZhou/13/2009(H3N2)(TZ13) used in this study was isolated from a patient in the Zhejiang Province of China, and was propagated in MDCK cells. This virus, termed TZ13, maintained a high titer in MDCK cells. The Influenza A/Puerto Rico/8/1934 (H1N1) virus was obtained by reverse genetics as previously described (Hoffmann and Webster 2000) using plasmids bearing the eight genes defined by accession numbers AF389115 to AF189122 in the NCBI Database.

Virus recovery

Template viral RNA was extracted from A/TaiZhou/13/2009(H3N2) using the QIAamp viral RNA extraction kit (Qiagen, Valencia, CA). The PB2, PB1, PA, NP, M, NS and NA genes of A/TaiZhou/13/2009 (H3N2) were reverse-transcribed using a reverse transcription kit (Biovisualab Ltd, Shanghai, China), the HA gene were synthetically produced according to the predicting results (Figure 2), and the HA sequence was confirmed by sequencing analyses. The cDNAs were amplified by HiFiFast DNA Polymerase (Biovisualab Ltd, Shanghai, China) using primers:

PB2 forward: 5'TATTggTCTCAgggAgCAAAAgCAggTC3',
PB2 reverse: 5'ATATggTCTCgTATTAgTAgAAACAAggTCgTTT3';
PB1 forward: 5'TATTCgTCTCAgggAgCAAAAgCAggCA3',
PB1 reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAggCATTT3';
PA forward: 5'TATTggTCTCAgggAgCAAAAgCAggTAC3',
PA reverse: 5'ATATggTCTCgTATTAgTAgAAACAAggTACTT3';
NP forward: 5'TATTCgTCTCAgggAgCAAAAgCAgggTT3',
NP reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAgggTATTTTT3';
NA forward: 5'TATTggTCTCAgggAgCAAAAgCAggAgT3',
NA reverse: 5'ATATggTCTCgTATTAgTAgAAACAAggAgTTTTTT3';
M forward: 5'TATTCgTCTCAgggAgCAAAAgCAggTAg3',
M reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAggTAgTTTTT3';
NS forward: 5'TATTCgTCTCAgggAgCAAAAgCAgggTg3',
NS reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAgggTgTTTT3').

Viruses were generated as described previously (Hoffmann and Webster, 2000). After confirming the sequences of all inserts by sequencing, the correct plasmids were used for virus recovery. Recovery of reassortant H3N2 influenza viruses was achieved by transfecting seven plasmid DNAs of TZ13 (TZ13-PB2, TZ13-PB1, TZ13-PA, TZ13-NP, TZ13-M, TZ13-NS and TZ13-NA), and plasmid encoding the different surface antigen genes of HA (PBR322-HA) (Figure 2) into 293T cells using Lipofectamine ™2000 (Invitrogen, CA). After 48 h, the transfected cells and supernatants were harvested for reassortant virus analysis. Briefly, the genes of the TZ13 virus were amplified by RT-PCR and inserted into plasmid pHW2000, virus stock were propagated in vero cell. Three reassortant influenza viruses were obtained and were termed rB, rD, rB/D influenza virus, rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions. The reassortant viruses rB, rD, rB/D were obtained using the reverse genetic method, in which the seven backbone fragments PB2, PB1, PA, NP, M, NS, and NA were derived from TZ13, whereas the HA surface antigen, B/D antigenic regions and other regions were synthesized in HA backbone fragment of TZ13.

Replication kinetics in MDCK cells

Multi cycle replication curves were generated according to inoculate

MDCK cell at a multiplicity of infection (MOI) of 0.01 and 50% tissue culture infectious doses (TCID50) per cell in duplicate. After inoculation, supernatants were sampled at 6, 12, 24 and 48 h, and virus titers in these supernatants were decided by means of end-point titration in MDCK cells.

Virus infectivity titration, TCID50

TCID50 (50% tissue culture infectious dose) titer was determined using the MDCK cells. Cells grown to the confluence in flat bottom 96-well plates were washed with PBS, and inoculated with serial 10-fold dilutions of the virus sample (diluted in full EMEM medium without FBS). Inoculated cells were incubated at 33°C, 5% CO_2 for 72 h. Cell infection in a given well was determined by cytopathic effect. TCID50 titers were calculated by the method of Reed and Muench (1938).

Pathogenicity of reassortant viruses in mice

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments described and approved by the Institute of the Fujian Medical University animals. To assess the pathogenicity of reassortant viruses, groups of eight 4-week-old BALB/c were inoculated with the reassortants viruses at a standard dose $10^{8.0}$ TCID50 by the intravenous route (i.v.) and observed for 14 days.

ELISA

The specific IgG and IgA titer against the reassortant viruses were determined by an indirect ELISA. Microtiter plates were coated with reassortant viruses (64 hemagglutinating units (HAU) per 50 ul) overnight at 4°C. Plates were washed with PBS-T buffer (PBS with 0.05%Tween 20) and blocked with (5%) skimmed milk diluted in PBS for 1 h at 37°C. Serum samples were diluted in dilution buffer (1% skimmed milk in PBS) and added to plates. Serum was two-fold serially diluted (starting from 1:10) and allowed to incubate for 2 h at 37°C. After washing in PBS-T, alkaline phosphatase-conjugated goat anti-mouse IgG, IgA (Sigma), diluted 1:1000 in PBS-T, was used as the detection antibody, with ρ-nitrophenylphosphate as a substrate (Sigma). The optical density (OD) was read at 405 nm using a Multiskan Ascent plate reader after substrate addition. Reciprocal serum antibody titers were calculated at 50% maximal binding on the titration curve. End point dilution titers were determined as the reciprocal dilution of the last well which had an OD_{405} above the mean OD_{405} plus two standard deviation of naive animal sera.

ELISPOT assays

Spleens from immunized mice were removed and single cell suspensions were prepared. Single cell suspensions of lymphocytes were suspended at 1×10^6 cells/200 ul in RPMI 1640 supplemented with (10%) heat-inactivated fetal bovine serum. The cells were cultured in triplicate and plated in enzyme-linked immunosorbent spot (ELISPOT) plates (BD Pharmingen) that have been previously coated with IL-4 or IFN-γcapture antibody (BD Pharmingen) overnight at 4°C, and then stimulated with variant virus at an MOI of 1. The spot-forming cells were detected by addition of biotinylated IL-4 or IFN-γ-detective antibody, followed by the addition of streptavidin-HRP and development with AEC substrate solution. Wells containing no antigen or 10 ug PMA were used as negative and positive control, respectively. Spots were counted using AID Immunospot (Cellular Technology Ltd.).

Cross-protection experiment

To evaluate the immunogenicity of reassortant viruses , 4-to 6-week-old female BALB/c mice (20 in each group) were immunized with two dose (days 0 and 14) of $10^{6.0}$ TCID50 by the intravenous route (i.v.). Mice were challenged intranasally with 20 μl containing $10^{8.0}$ TCID50 of A/PR/8/34 and TZ13 monitored for death or survival over a period of 14 days.

Statistical and bioinformatics analyses

Data was evaluated using GraphPad Prism 5 software. The statistical significance of difference was evaluated by two-ANOVA. A p value of $p<0.05$ was considered significant. The nucleotide sequences determined in this study was available from GenBank under Sequin numbers DQ017486.

RESULTS

Selection of H3N2 influenza vaccine strains

Seasonal H3N2 influenza vaccine strains full-length HA protein sequences that were unrepeated from Influenza Research Database over the past twenty years (1990-2012) from the Northern hemisphere was selected. Based on the above criteria, six H3N2 influenza vaccine strains were discovered. Also, the amino acid changes in the HA proteins of the H3N2 influenza vaccine strains were compared, and it was found out that there were 54 amino acids changes in the HA proteins. The amino acids changes were concentrated on the HA1 region, which was 45/54. The HA1 region was found variable, while HA2 region was conserved over the entire evolution of the H3N2 viruses within the study period (1990 -2012).

Predicted conserved hemagglutinin CD4+ T and CD8+ T cells epitopes on H3N2 influenza vaccine strains

In order to have a better understanding of the antigenic changes of seasonal influenza vaccine strain H3N2, the gene sequence was analyzed and the HA proteins for the four antigenic regions: A,B,C,D in H3 HA molecules were compared (Figure 1). During the period of twenty years, it was discovered that amino acids changes were mainly in B/D antigenic regions. So, B/D regions were selected to predict the hemagglutinin CD4+ T and CD8+ T cells epitopes and only one group of B/D antigenic regions was decided on. The predicted epitopes of the B/D group exhibited strong MHC-Ⅰ/MHC-Ⅱbinding affinity. The strong binding affinity thresholds were >500 nM. The degree of predicted epitope was validated on protective immune with the experiments. Decision on T cell epitopes in highest binding affinity of the B and D antigenic regions

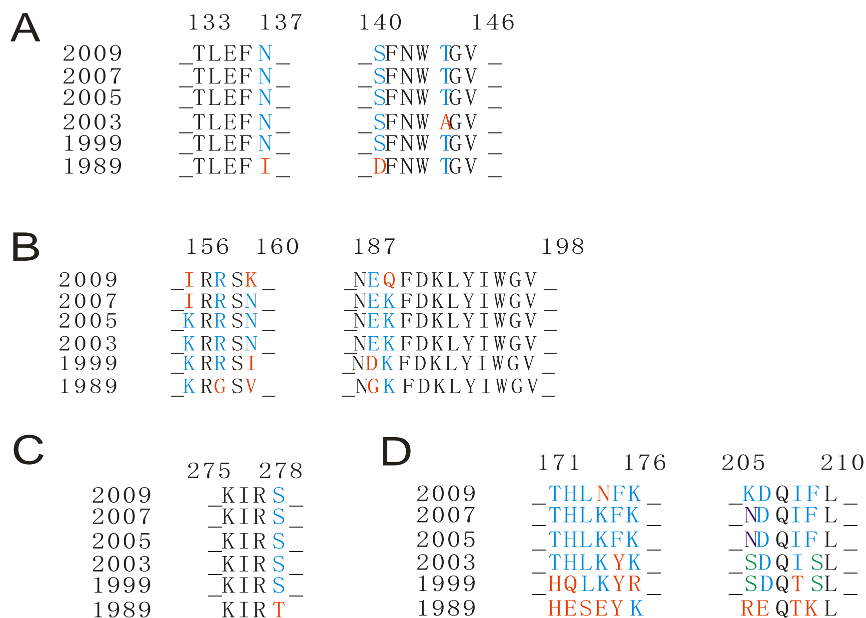

Figure 1. The analysis and comparisons of the HA protein of H3N2 seasonal influenza vaccine strains from Influenza Research Database over the past twenty years (1990-2012) from the Northern hemisphere.

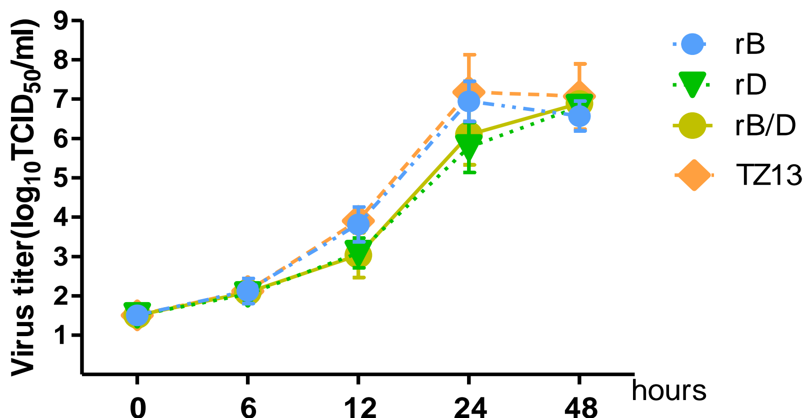

Figure 2. Growth kinetics of reassortant viruses in MDCK cells. Mean and standard deviations of three repeat assays are shown. The viral titer was detected by TCID50. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

of the HA protein, and the amino acid position of B region (156-160,187-198) were KRRSN and NEQFDKLYIWGV, the amino acid position of D region (167-182, 201-215) were LNKRQHSNEKYPALNV and PGTDNDQTELYAQAS.

Growth kinetics of reassortant viruses

All the reassortant viruses replicated to a similar extent at 6, 12, 24, 48 h (Figure 2). At 48 h, the titer of reassortant virus was 107.0TCID50. These results show that, the growth kinetics of reassortant viruses and the reassortant viruses obtained has similar ability for growth in mammalian cells.

Pathogenicity of reassortant viruses in mice

To further investigate the potential effects of mutations on pathogenicity, the context of the mouse model was

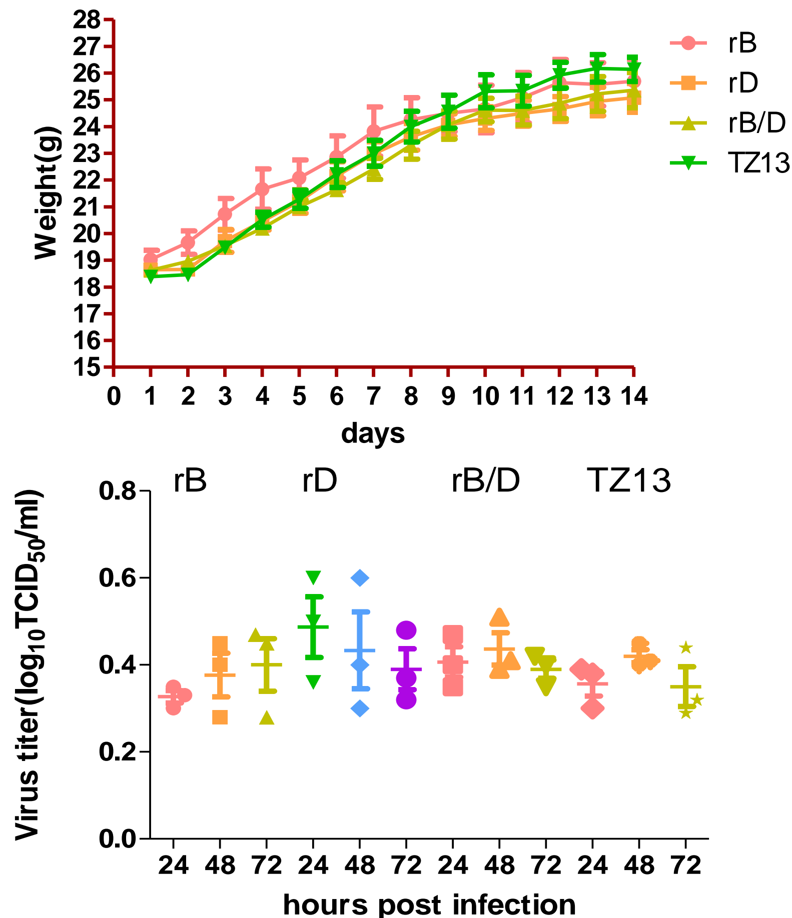

Figure 3. Changes of B/D antigenic regions in the HA protein increase weight and did not increase viral replication in mice. Mice were infected by the i.v., and weight loss (A) was monitored. (B) Lung tissue was collected from mice (n=3) at different time points after infection and homogenized, and viral titers were determined by TCID50. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

characterized. Groups of eight-week-old females were inoculated at a standard dose $10^{8.0}$ TCID50 by the intravenous route (i.v.) and then monitored for weight loss (Figure 3) and survival daily for 14 days to assess the effects of the infection. All mouse remained healthy throughout the 14-day observation period with no mortalities. Mice weight and behavior were recorded and observed daily for 14 days as shown in Figure 3, the body weights of the four groups were very similar to that of the weight group. The weight increased from 18.3±0.5 g at day 1 to 26.8±1.5 g at day 14.

In a subsequent experiment, mice infected with the same amount of the respective viruses were euthanized at the indicated time points and viral titers in the lung were determined by TCID50 (Figure 3). As expected, mice infected with reassortants viruses displayed low titer at all time points. These results demonstrate that reassortant viruses have a low pathogenicity similar to that of A/nan chang.

Humoral immune responses in mice

To assess the ability of the reassoratants viruses to induce virus specific immunity against influenza virus, 4- to 6-week-old female BALB/c mice (20 in each group) were immunized twice (days 0 and 14) with one dose of $10^{6.0}$ TCID50 by the intravenous route (i.v.). Three weeks after the final vaccination, to examine virus-specific antibody production, IgG and IgA in the nasal wash, BAL and serum of immunized mice were measured by using ELISA (Figure 4). As shown in Figure 4, when compared with the wt group, vaccination with rB/D virus induced siginificantly strong antibody response in BAL and serum. Notably, both the virus-specific IgG and IgA titer in BAL, and the IgG in serum from mice immunized with rB/D

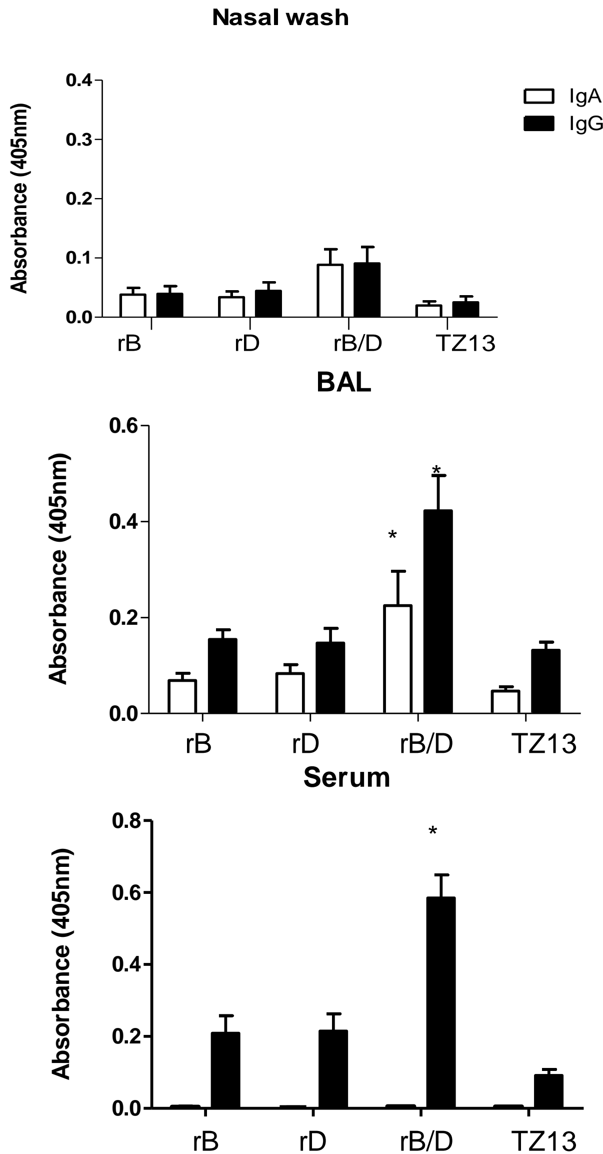

Figure 4. Induction of virus-specific IgG and IgA in nasal wash, BAL and serum of mice immunized with reassortant viruses. Virus-specific antibodies were detected by means of an ELISA. Sample (n=5) from each group were obtained 21 days after the vaccination. Results are expressed as the mean and standard deviations absorbance of 1:10 diluted samples (serum). Statistically significant differences among the groups were assessed by TWO-ANOVA (*P<0.05). rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

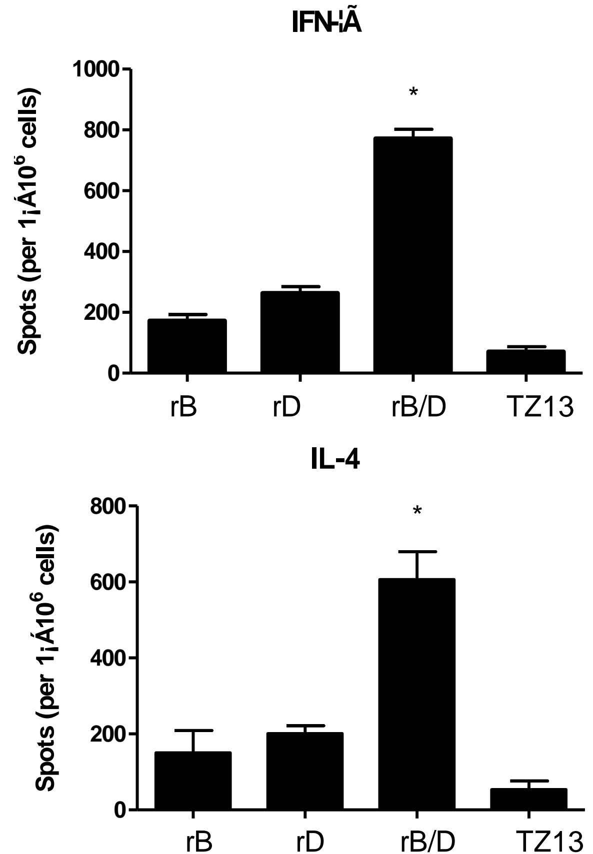

Figure 5. The level of IFN-γκand IL-4 spot-forming cells in spleens of mice as determined by ELISPOT assays. Mice were immunized and splenocytes were isolated and stimulated with reassortant viruses. Bars represent mean and standard deviations of spot counts in triplicate wells. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

reassortant virus were higher than the respective titers from mice immunized with other three influenza viruses.

T helper cell responses in mice

To investigate the T helper cell responses, splenocytes were prepared on day 21 and stimulated with the reassortant viruses *in vitro*. The numbers of INF-γand IL-4 producing cells were determined by ELISPOT assays. The results indicated a mixed Th1 (INF-γ) and Th2 (IL-4) response was observed (Figure 5). TH-1 and TH-2 type responses were found to be predominant at rB/D group. IL-4 and INF-γresponses were relatively higher in mice immunized rB/D influenza virus. These results demonstrate that rB/D influenza virus can improve the higher immunity than other reassortant influenza viruses in mice.

Cross- protection studies in mice

BALB/c mice were used for challenge and protection studies. Two weeks after the boost immunization, all animals were challenged with the heterologous PR8. Challenge with the PR8 resulted in a transient body

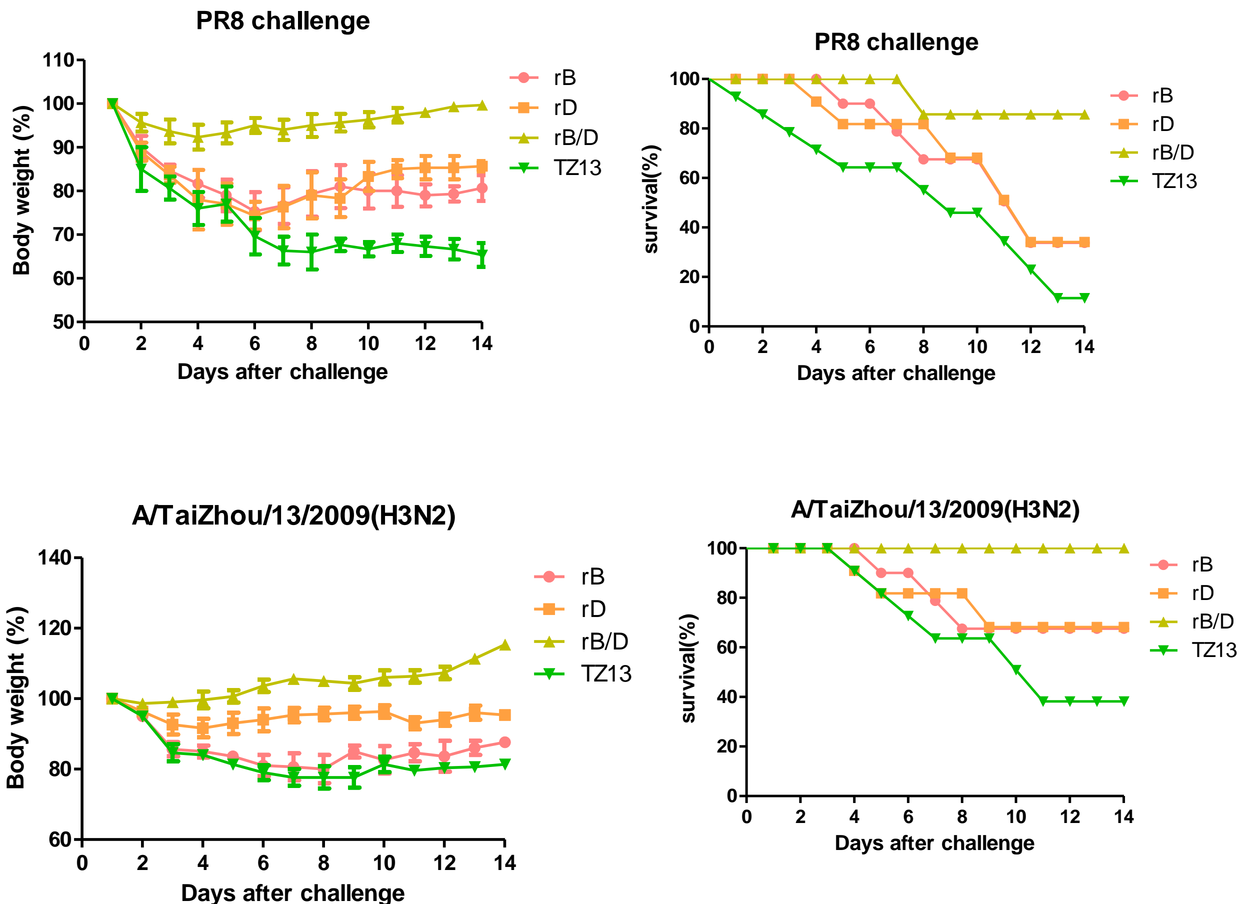

Figure 6. Body weight changes and survival rates of mice after different lethal influenza virus challenge. A body weight changes and survival rates of mice after challenge with the PR8; B body weight changes and survival rates of mice after challenge with the TZ13. Each point represents the mean of five mice on each day. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

weight loss at day 7, recovery on the following 7 days, and rB/D group has the lowest body weight loss (10%) and the highest survival (80%) among reassortant influenza viruses groups, whereas the other groups, 40% were dead. There was significant difference between the rB/D group and other three groups ($P<0.05$). These results indicated that rB/D influenza virus is the most effective to protect mice against heterologous lethal influenza virus challenge.

DISCUSSION

Influenza vaccine strains must be selected each year by WHO collaborating centers because influenza A viruses undergone sufficient antigenic drift to evade existing antibody responses (Wright et al., 2006). Influenza virus hemagglutinin is important for virus virulence, sometimes, only one amino acid substitution often interferes with virus virulence and resistance (Chen et al., 2010; Liu et al., 2010). HA protein of influenza virus is a main component of influenza vaccine. Based on HA protein, there were amounts of influenza vaccines studies (D'Aoust et al., 2010; Steel et al., 2010). Owing to the similarity and specificity of HA protein in different types and subtypes, cross-reactive antibodies usually appear to protect against different influenza virus infections (Wrammert et al., 2011). Although, functional antibody responses are an accepted correlate for vaccine induced protection, there are increasing reports that T-cell responses are important.

Reverse genetic technology, is used to generate influenza virus from cells co-transfected with plasmids of influenza virus gene segments, and has been applied to vaccine development since 1998. The influenza A H3N2 virus A/Taizhou/13/2009 (TZ13) was isolated from a patient in Hangzhou of China in 2009. In this study, the backbone from TZ13 was employed using reverse genetics and some reassortant viruses were generated. The HA protein segments of reassortant viruses were predicted from T cell epitopes according to the changes of B and D antigenic regions of the HA of the influenza

vaccine strains for the past twenty years. The reassortant vaccine viruses were generated in MDCK cells. Then, their immunogenicity and efficacy in animal models was evaluated. The reassoratnt viruses were attenuated in mice. After immunization, rB/D influenza virus induced both humoral and cell immune responses in mice and completely protected these animals from challenge with PR8 virus. Both the virus–specific IgG and IgA titer in BAL, and the IgG in serum from mice immunized with rB/D reassortant virus were higher than the respective titers from mice immunized with other three influenza viruses. TH-1 and TH-2 type responses were found to be predominant at rB/D group. IL-4 and INF-γresponses were relatively higher in mice immunized rB/D influenza virus. This report mainly focused on obtaining high binding affinity T cell epitopes of a reassortant influenza virus, current research put emphasis on the immunicity and cross-protection of the reassortant virus.

Conclusion

The present study demonstrates that reassortant influenza virus based on HA protein changes of influenza vaccine strain could be used to produce effective cross-immunicity. These results demonstrate that the rB/D influenza virus could elicit humoral immunity more efficiently. Comparably, these datas on mice support the evaluation of this vaccine. It is important for a suitable match in the antigenicity of pandemic strain, according to these properties to produce vaccine in the face of an influenza pandemic.

Competing interests

The authors' declare that they have no competing interests.

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Full Length Research Paper

Detection of virulence and antibiotic resistance genes in environmental strains of *Vibrio* spp. from mussels along the coast of Rio de Janeiro State, Brazil

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Mussels have a filter system enabling them to take up nutrients from the water, so a microbiological analysis of these bivalve mollusks can show the contamination levels of their surrounding aquatic environment. The present work aimed to isolate *Vibrio* species from two hundred samples of mussels (*Perna perna*) incrusted on rocks of the Santana Archipelago and from longline mariculture in Ilha Grande Bay in Angra dos Reis and from Arraial do Cabo, all of which are in Rio de Janeiro state, Brazil. A total of 209 *Vibrio* were isolated. The most prevalent species was *Vibrio parahaemolyticus* (44.66%) followed by *Vibrio alginolyticus* (19.62%) and *Vibrio vulnificus* (12.44%). All 209 *Vibrio* isolates tested positive for the RNA polymerase alpha gene (*rpoA*). The *tlh* gene (thermolabile hemolysin), a genetic marker for *V. parahaemolyticus*, and *vvhA* (cytolysin hemolysin) of *V. vulnificus* were detected in 85 and 26 isolates, respectively. The MALDI-TOF MS proteomic technique was used to confirm the identification of the 41 *V. alginolyticus* isolates. Our most important finding was the detection of the *tdh* virulence gene in 68.20% (58/85) of *V. parahaemolyticus* environmental strains. Besides the circulation of the virulence gene, the spread of antimicrobial resistance was evaluated and 91.3% (191/209) of the isolates showed resistance to ampicillin, 23.9% (50/209) to ciprofloxacin, 18.6% (39/209) to nitrofurantoin, 5.7% (12/209) to tetracycline, 4.3% (9/209) to pefloxacin and 3.3% (7/209) to chloramphenicol. These findings indicate that environmental isolates can act as reservoirs of virulence and antibiotic resistance genes.

Key words: *vvh*A gene, mussels, public health, *rpoA* gene, *tlh* gene, *Vibrio.*

INTRODUCTION

Mussels are filter-feeding bivalve organisms that pump seawater through their digestive systems to obtain oxygen and food. They thus accumulate and concentrate both harmless as well as pathogenic microorganisms,

such as human enteric viruses and the pathogenic *Vibrio* species. So, the microbiological quality of bivalve mollusks is directly related to the environmental quality of their aquatic surroundings. Furthermore, due to their distribution along coastlines and in estuaries, these mussels are often exposed to sewage pollution, mainly in areas near big cities (Asplund, 2013).

The consumption of raw or undercooked marine bivalves, which is a common practice in Brazil, poses a potential health hazard for humans due to the possible ingestion of pathogens. Besides this potential of food-borne diseases, the sessile characteristic of mussels enables the encrustation of various sites, such as coastal rocks and cables. This represents a health risk to fishermen and handlers of underwater cables because they can be injured by the hard shells and become infected with any of the pathogenic bacteria found in these animals or from the water (Sousa et al., 2004; Pereira et al., 2007).

The marine bacteria genus *Vibrio* is widespread in tropical coastal waters worldwide. Pathogenic and nonpathogenic species are widely distributed, particularly in estuarine waters, where they accumulate in edible animals (Yeng and Boor, 2004; Pereira et al., 2007). Some *Vibrio* isolates are clinically significant in humans, and others are known to cause diseases in fish (Vieira et al., 2010). The main pathogenic species to humans are *V. cholera*, *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* (Thompson et al., 2004). In the present study, *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* were isolated from mussels incrusted on rocks of the Santana Archipelago and from longline mariculture in Ilha Grande Bay located in Angra dos Reis and Arraial do Cabo, all of which are important sites of marine activity in Rio de Janeiro state, Brazil. So due to their importance as food-borne pathogens and infections of tissue injuries we decided to investigate them as potential reservoirs of virulence and antibiotic resistance genes.

Vibrio parahaemolyticus is an autochthonous bacterium prevalent in marine and estuarine environments worldwide (Joseph et al., 1982). While the majority of environmental strains are innocuous members of the marine microbiota, small subpopulations are opportunistic pathogens for humans, associated with gastroenteritis (Johnson et al., 2008) and extra-intestinal infections such as secondary septicemia, ocular infections and otitis, besides contaminating open wounds after exposure (Drake et al., 2007). Potentially virulent strains are commonly differentiated from likely avirulent strains by the presence of the thermostable direct (*tdh*) and/or *tdh*-related (*trh*) hemolysin genes (Bej et al., 1999). Virulent strains are also able to produce thermolabile hemolysin (TLH), encoded by the *tlh* gene. The *Tlh* gene is not associated with pathogenicity, since it has been observed in all clinical and environmental *V. parahaemolyticus* strains, and is considered a species-specific marker which is employed in *V. parahaemolyticus* characterization studies (Ward and Bej, 2006; Rojas et al., 2011). Although there is widespread belief that environmental isolates lack the virulence genes usually found in clinical strains, some studies indicate that virulence genes, or their homologues, can also be present in strains from environmental sources and that acquisition of such genes might take place in the aquatic environment (DePaola et al., 2003; Nordstrom et al., 2007).

Vibrio vulnificus is a virulent pathogen that causes two distinct major conditions: Primary septicemia, caused by the consumption of raw or undercooked seafood, and necrotizing wound infections, acquired through the exposure of an open wound to warm seawater with high concentrations of *V. vulnificus* (Bross et al., 2007). In addition, *V. vulnificus* has also been associated with other clinical disorders, including pneumonia, osteomyelitis, spontaneous bacterial peritonitis, eye infections, and meningitis (Pfeffer et al., 2003). Hemolytic/cytolytic exotoxin has been the most studied virulence marker. This toxin, encoded by a gene known as *vvhA* or *cth*, is a heat-labile enzyme that lyses mammalian erythrocytes and is cytotoxic to a variety of mammalian tissue culture cell lines (Drake et al., 2007).

Vibrio alginolyticus is commonly found in marine environments. It is recognized as an opportunistic pathogen to both humans and marine animals (Campanelli et al., 2008). Rising ocean temperatures have led to reports of out-of-season *V. alginolyticus* outbreaks in the United States, Spain, Mexico, Japan and China (Sganga et al., 2009; Yoder et al., 2008; Cavallo and Stabili, 2002). The pathogenesis and epidemiology of *V. alginolyticus* infections are still unclear (Wang et al., 2008).

The great variability of the biochemical characteristics of *Vibrio* species makes it difficult to standardize an accurate phenotypic identification (Thompson et al., 2004; Tarr et al., 2007). Consequently, more specific, rapid and sensitive molecular methods for the identification of *Vibrio* species must be developed. A PCR based on the RNA polymerase alpha subunit (*rpo*A) gene has been used for the detection of the *Vibrio* genus (Dalmasso et al., 2009). Also, virulence genes related to some *Vibrio* species have been used for species identification (Panicker et al., 2004; Rojas et al., 2011). Additionally, as antimicrobial resistance has been a growing public health challenge (Chai et al., 2008), the antimicrobial resistance profile of the *Vibrio* isolates was

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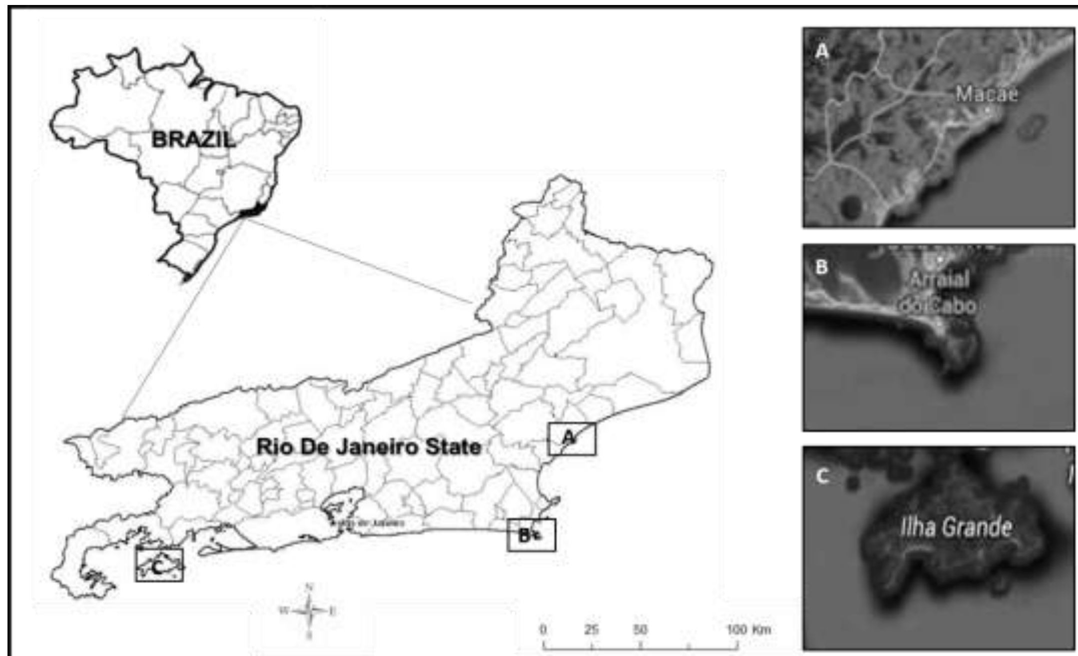

Figure 1. Map showing the locations where the mussels were collected; (A) Santana Archipelago, Macaé, (B) Arraial do Cabo Beach and (C) Ilha Grande Bay located in Angra dos Reis, Rio de Janeiro State, Brazil.

monitored to understand their possible role as resistance gene reservoirs. Zulkifli et al. (2009) stated that food contamination with antibiotic-resistant bacteria is a threat to public health, since the resistance determinants can be transferred to other bacteria of clinical significance, and that *Vibrio* isolates are candidate vehicles for such transfer because of their diversity and because they can survive in the gastrointestinal tracts of both humans and animals.

The inappropriate use of antibiotics in aquaculture is one of the causes for the high incidence of antimicrobial resistant bacteria isolated from aquatic environments that represent a danger for aquatic organisms and human health (Rebouças et al., 2011). Also, the discharge of untreated industrial and domestic waste in the aquatic environment contributes to spread of resistance (Sotomayor and Balcázar, 2003). Although the Brazilian seacoast is very extensive, there is little information available on the level of antimicrobial resistance in the pathogenic bacteria from this aquatic environment.

Accordingly to the aforementioned issues, the present work aimed to characterize the virulence and resistance profiles of the most prevalent species of *Vibrio* isolates isolated from mussels along the seacoast of Rio de Janeiro state, Brazil.

MATERIALS AND METHODS

Sampling

Mussels of the *Perna* species were collected at three different times and places in the state of Rio de Janeiro. In total, seven samples were taken: Three were from the rocks of the Santana Archipelago, offshore from Macae, from June 2007 to May 2008 where the animals were taken directly from the rocks, at depths ranging from 3 to 4 m by divers. The geographic coordinates of the collection points were: 22º 23' 45.43'' S - 41º 43' 42.52'' W. Two other samples were taken from mariculture longline in the Bay of Ilha Grande facing the town of Angra dos Reis in October 2010 and February 2011 between latitudes 22° 55' S and 23° 15' S and longitudes 44° 43' W and two collections were made on the beach in Arraial do Cabo in May and July 2011 at 22° 56' 31" S and 42° 8' 19" W (Figure 1). At each sampling, 2 lots of 25 adults mussels were collected, with closed valves and the same size as used commercially (greater than 6 cm). The mussels were whashed individually with brush in running drinking water to remove the dirt. During this process were rejected the animals with open valves, totalizing two hundred samples of mussels (*Perna perna*). The samples were placed in polyethylene boxes and transported at 6 to 10°C to the Veterinary Bacteriology Laboratory of Federal Rural University of Rio de Janeiro.

Microbiological analysis

The body mass and intravalvular liquid was collected in beaker, where he was held crushing of the solid parts in order to promote homogenization of the material. In order to detect Vibrio spp., 25-g of the sample was added to 225 ml of alkaline peptone water (APW) containing 1% NaCl. Serial dilution of each sample was performed until 10^{-2} dilution in APW and was incubated at 37°C for 18 h. After that, the samples were inoculated in dishes containing Thiosulfate Citrate Bile Sucrose agar (TCBS) with the addition of 1, 2 and 3% NaCl and incubated at 37°C for 18 to 24 h (Konemam et al., 2008). Five to ten colonies from each dish were inoculated into tubes containing nutrient agar, LIA (lysine iron agar) and KIA (Kligler iron agar), along with 1% NaCl and incubated at 37°C for 24

h in order to carry out the differential diagnosis between *Vibrio* isolates and members of the Enterobacteriaceae family. The isolates were also submitted to the cytochrome-oxidase test to distinguish between the *Vibrio* isolates and members of the Enterobacteriaceae family (Koneman et al., 2008). *Vibrio* species identification was performed through biochemical tests based on the vibriostatic agent O/129 resistance (2,4 diamine-6,7 diisopropyl pteridine); production of ortho-nitrophenyl-β-galactoside (ONPG); production of acetoin by using the Voges-Proskauer test; glucose, sucrose, arabinose and mannose fermentation; lysine and ornithine decarboxylase; arginine dehydrogenase; halophilia production in different NaCl APW concentrations (0, 3, 6, 8 and 10%); motility observation, indole production; and nitrate reduction (Sewell, 2002).

Detection of *rpoA, tlh, tdh, trh* and *vvhA* genes

Bacterial DNA extraction was performed by thermal shock according to the method proposed by Dalmasso et al. (2009). A pair of primers that targets *rpo*A (Dalmasso et al., 2009), *tlh (*Bej et al., 1999), *tdh* (Honda and Iida, 1993; Frakuddin et al., 2012), *trh* (Honda and Iida, 1993) and *vvhA* (Brauns et al., 1991) genes was used to amplify DNA from *Vibrio* isolates and PCR conditions were performed as described previously by the authors mentioned above. Reference strains were kindly provided by Oswaldo Cruz Foundation - Fiocruz, Brazil: *V. parahaemolyticus* (ATCC 17802) was used as positive control in detection of genes *rpo*A, *tlh* and *tdh* (Frakuddin et al., 2012), *V. alginolyticus* (ATCC 17749) to detection of gene *rpo*A, and *V. vulnificus* (ATCC 27562) to detection of genes *rpo*A and *vvh*A.

Vibrio alginolyticus identification by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)

To perform the MALDI-TOF MS, the samples were inoculated in BHI agar at 37°C for 24 h. Each culture was transferred to a microplate (96 MSP, Bruker - Billerica, USA). Each bacterial sediment was covered by a lysis solution (70% formic acid, Sigma-Aldrich). A 1-µL aliquot of matrix solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich). The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker) equipped with a 337-nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 (Bruker) program. The spectra were collected in a mass range between 2,000 and 20,000 m/s, and then were analyzed by the MALDI Biotyper 2.0 (Bruker) program, using the standard configuration for bacteria identification, by which the spectrum of the sample was compared with the references in the database. The results are given on a 0-3 scale, where the highest value means a more precise match and reliable identification. In this study, we accepted values for matching greater than or equal to two (2) as proposed by the manufacturer.

Antimicrobial susceptibility tests

The isolates were submitted to susceptibility tests by the disk diffusion technique, as standardized by CLSI (2011). The following antimicrobial disks were used: (SENSIFAR-Cefar): tetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), pefloxacin (5 µg), ampicillin (10 UI) and ciprofloxacin (5 µg). After 24-h incubation at 35°C, the diameters of the inhibition zones were measured in millimeters and analyzed by the interpretation criteria established in the CLSI standards (2011).

RESULTS AND DISCUSSION

A total of 209 *Vibrio* isolates were phenotypically characterized. The prevalent species were *V. parahaemolyticus* (n=85), *V. alginolyticus* (n=41) and *V. vulnificus* (n=26). We also detected *V. harveyi* (n= 13), *V. fischerii* (n=2), *V. anguillarum* (n=2) and *V. carchariae* (n=3) (Table 1).

All 209 isolates tested positive for *rpo*A gene yielding a 242-bp fragment (Figure 2). The *rpo*A gene detection is considered a specific, sensitive and fast method for *Vibrio* identification (Thompson et al., 2005; Dalmasso et al., 2009; Jeyasekaran et al., 2011).

The great variability of biochemical characteristics of *Vibrio* species makes it difficult to standardize an accurate phenotypic identification (Thompson et al., 2004; Tarr et al., 2007). So the prevalent species *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* were submitted to genotypic characterization in order to confirm the previous phenotypic identification.

The *tlh* (thermolabile hemolysin) is a species marker gene for *V. parahaemolyticus* (Rojas et al., 2011). A total of 85 isolates was classified as *V. parahaemolyticus* since they tested positive for *tlh* amplification yielding a 450-bp specific fragment (Figure 3). The *rpo*A gene (242-bp) was used as endogenous control.

All the 124 isolates (59.3%) that tested negative for the *tlh* gene were submitted to a PCR for the *vvh*A (cytolysin-hemolysin) gene, a species marker gene for *V. vulnificus* (Drake et al., 2007). Twenty-six isolates (20.9%) tested positive for *vvhA* gene yielding a 386-bp fragment (Figure 4). Although widely used and highly specific to *V. vulnificus*, this gene is not able to predicate the virulence potential of *V. vulnificus* strains, since there are other virulence mechanisms associated with *V. vulnificus*, such as the presence of the polysaccharide capsule, protease, elastase and phospholipase, which may be found in almost all clinical and environmental strains (Who, 2005). The close relation of *V. alginolyticus* to other species such as *V. parahaemolyticus* and the absence of a reliable specific genetic marker require additional techniques for its differentiation. Therefore, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for *V. alginolyticus* identification. This technique is based on proteomic technology and is considered a more specific, rapid and sensitive method. MALDI-TOF MS was a useful tool for reliable identification of *V. alginolyticus,* confirming the phenotypic identification of all 41 isolates. Several authors concluded that the whole-cell MALDI-TOF mass spectrometry analysis is a highly reproducible method for rapid discrimination of *Vibrio* isolates and related species including *V. alginolyticus* (Dieckmann et al., 2010; Hazen et al., 2009).

The spatial distribution of *V. parahaemolyticus* agrees with the important epidemiologic role due to the

Table 1. Phenotypic characterization percentage of *Vibrio* isolates from different harvesting sites according to number of isolates

Vibrio species	Phenotypic characterization % (number of isolates)			
	Macaé	Arraial do Cabo	Angra dos Reis	Total
V. harveyi	8(8)	3.4 (2)	5.8 (3)	6.2 (13)
V. vulnificus	20 (20)	5.1 (3)	5.8 (3)	12.4 (26)
V. carchariae	1 (1)	1.7 (1)	1.9 (1)	1.4 (3)
V. parahaemolyticus	44 (44)	31.0 (18)	45.0 (23)	40.6 (85)
V. alginolyticus	15 (15)	18.9 (11)	29.4 (15)	19.6 (41)
V. damsela	2 (2)	-	1.9 (1)	1.4 (3)
V. aestuarianus	-	1.7 (1)	-	0.4 (1)
V. costicola	-	1.7 (1)	1.9 (1)	0.9 (2)
V. cincinnatiensis	-	1.7 (1)	-	0.4 (1)
V. fischeri	-	1.7 (1)	1.9 (1)	0.9 (2)
V. anguillarum	2	-	-	0.9 (2)
V. metschnikovii	-	1.7 (1)	-	0.4 (1)
V. mimicus	-	1.7 (1)	-	0.4 (1)
Vibrio spp.	8 (8)	29.3 (17)	5.8 (3)	13.3 (28)
Total of isolated	100	58	51	290

-, detected.

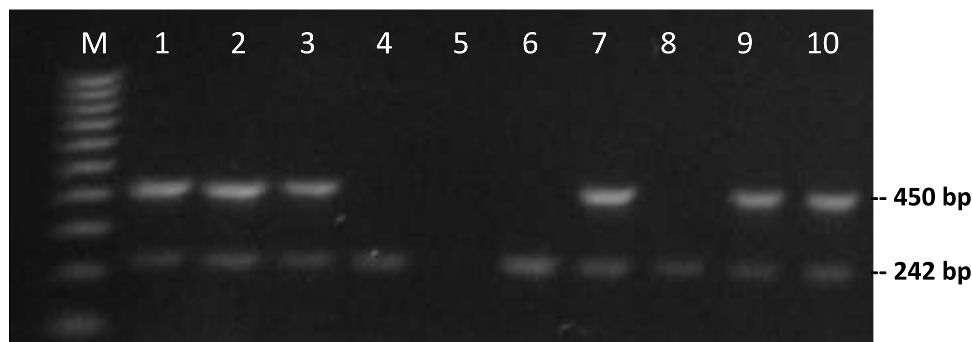

Figure 2. Amplification of *rpoA* and *tlh* genes in *Vibrio* spp. M: 100-bp ladder, 1: ATCC 17802 *Vibrio parahaemolyticus*; 2, 3, 7, 9, 10: *V. parahaemolyticus*; 6: *V. vulnificus*; 5: negative control.

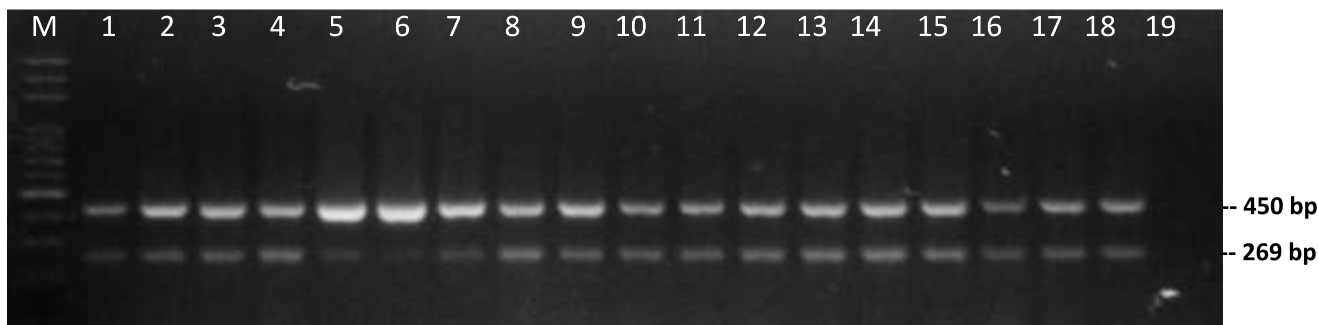

Figure 3. Amplification of *tdh* gene in *V. parahaemolyticus*. Internal control: *tlh* gene. M: 100 bp DNA Ladder, 1-18: *V. parahaemolyticus strains*; 19: negative control.

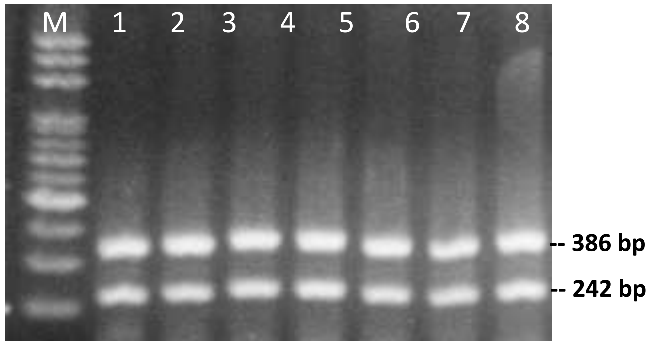

Figure 4. Amplification of *rpoA* and *vvhA* genes in *V. vulnificus*. M: 100-bp ladder; 2: ATCC 27562 *V. vulnificus*, 3, 4, 5, 6, 7; 8: *V. vulnificus*.

occurrence of epidemic outbreaks of this agent after the ingestion of either raw or undercooked seafood (Cabrera-Garcia et al., 2004). Similarly, *V. alginolyticus* was the second most isolated species, which reinforces its epidemiological importance, associated with the occurrence of diarrhea and wound infections, particularly among mussel handlers (Rodrigues et al., 2001). The detection of 26 *V. vulnificus* isolates represented an important microbiological finding, since this species is characterized as highly pathogenic to humans (Nascimento et al., 2001).

Although some authors have considered that environmental *V. parahaemolyticus* strains lack the thermostable direct (*tdh*) and *tdh*-related (*trh*) hemolysin virulence genes (Su and Liu, 2007; Rojas et al., 2011), we decided to investigate their presence in order to establish the circulation of these genes in the aquatic environment. Surprisingly, 58 *V. parahaemolyticus* strains (68.2%) tested positive for *tdh* gene (58/85) yielding a 386-bp fragment (Figure 4). The *tlh* gene was used as the endogenous control. However, the *trh* gene was not detected. Virulent strains can present both *tdh* and *trh* genes or just one of them (Su and Liu, 2007; Rojas et al., 2011).

According to Bhoopong et al. (2007), only 1 to 2% of environmental strains present these virulence genes. These data show the prospective occurrence of virulence genes circulating in aquatic environments, since most of the reported data in the literature have been obtained from clinical specimens. Our findings are similar to those of DePaola et al. (2003) and Nordstrom et al. (2007), who considered the recent detection of virulence genes in environmental *V. parahaemolyticus* strains is a consequence of the employment of more sensitivity molecular methods. Caburlotto et al. (2008) reported for the first time the pandemic potential of a *V. parahaemolyticus* strain presenting both *tdh* and *trh* genes from marine water in Caleri, Italy. These authors emphasized the marine environment as a reservoir of pandemic strains and virulence genes (Caburlotto et al., 2010). As far as we know, this is the first time that the *tdh* gene has been detected in *V. parahaemolyticus* environmental strains along the coast of Rio de Janeiro State. The observed *tdh*+ *V. parahaemolyticus* strains in our study can be associated with different sources of human and animal fecal contamination of seawater. In Arraial do Cabo, there is a floating restaurant nearby the collection sites. Similarly, in Ilha Grande Bay, in Angra dos Reis, domestic effluent is discharged without previous treatment into the bay. And although the Santana Archipelago is well offshore, there are oil platforms and plenty of seabirds that can also contribute to fecal contamination. Cabrera-Garcia et al. (2004) suggested that fecal contamination can be the origin of the *tdh*+ environmental strains by genetic material exchange between pathogenic and environmental strains. Besides virulence gene circulation, the spread of antimicrobial resistance is a challenge of utmost importance. The evaluation of the susceptibility profile of the 209 *Vibrio* strains showed 91.3% (191/209) resistance to ampicillin, 23.9% (50/209) to ciprofloxacin, 18.6% (39/209) to nitrofurantoin, 5.7% (12/209) to tetracycline, 4.3% (9/209) to pefloxacin and 3.3% (7/209) to chloramphenicol (Table 2). These data are of significant concern since *Vibrio* is usually considered highly susceptible to virtually all antimicrobials. *Vibrio* isolates live in coastal and estuarine waters, open areas particularly subject to environmental contamination by agricultural runoff or wastewater, which may contain significant levels of antimicrobials and heavy metals, exerting selective pressure for the development of antimicrobial-resistant aquatic bacteria (Han et al., 2007). One of the main findings in this study was the high ampicillin resistance among *Vibrio* isolates from all collection sites. Recently, Jun et al. (2012) reported the detection of beta-lactamase genes carried by the ampicillin-resistant *V. alginolyticus*, *V. cholerae* and *Photobacterium damselae* subsp. *damselae* strains isolated from marine environments with the primers designed from a novel beta-lactamase gene cloned from *V. alginolyticus* KV3, isolated from aquaculture water of Geoje Island, Korea. This information points to the possibility of developing new strategies for ampicillin resistance that need to be investigated further to confirm or refute the idea of the emergence of resistant *Vibrio* isolates in aquaculture (Han et al., 2007).

The resistance to ciprofloxacin was detected in both the Arraial do Cabo and Angra dos Reis samples. As in other gram-negative bacteria, resistance to fluoroquinolones in *Vibrio* isolates is mostly mediated by mutations within the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. This mutational change was present in the majority of fluoroquinolone-resistant *Vibrio* isolates and probably can explain the resistance detected in the present study.

The emergence of multi-drug resistant bacteria in recent years is worrying and is eroding the antibiotic

armamentarium and thus limiting the therapeutic options available to clinicians (Zulkifli et al., 2009). Considering this, frequent and careful monitoring of antibiotic use in aquaculture, for the purpose of preventing and treating microbial infections or as animal growth promoters, is important to ensure aquatic environmental safety (Noorlis et al., 2011).

The results of this study show the detection of the *tdh* virulence gene in 68.2% (58/85) of *V. parahaemolyticus* environmental strains and the high rate of ampicillin resistance among *Vibrio* isolates from all the collection sites. These findings indicate that environmental strains can act as reservoirs of virulence and antibiotic resistance genes. The detection of *rpoA* and *tlh* genes yielded reliable genus confirmation and *V. parahaemolyticus* identification, respectively. Also, the MALDI-TOF MS technique was very efficient in distinguishing *V. alginolyticus* from the closely related species *V. parahaemolyticus*.

Conflict of Interests

The author(s) declare there are no conflicts of interests.

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