African Journal of Microbiology Research

Volume 8 Number 35, 27 August, 2014
ISSN 1996-0808

Academic Journals

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academicJournals

Vol. 8(35), pp. 3216-3219, 27 August, 2014
DOI: 10.5897/AJMR2014.7004
Article Number: 5A0196047142
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Avian ochratoxicosis: A review

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Received 10 July, 2014; Accepted 22 August, 2014

Ochratoxicosis is one of the most common mycotoxicoses in poultry, specially commercial poultry. It is caused by most dangerous mycotoxin because it causes oncogenic effects in embryos, that is, ochratoxin A. The presence of ochratoxin-A in poultry feed contributes significantly to health disorders and decreases production. This is one of the causes of economic losses in poultry industry due to increased mortality, reduced body weight gain, reduction of carcass quality, greater feed conversion rate and immunosuppression. The risk associated with ochratoxin residues in poultry meat represents a public health concern. The present article reviews most significant scientific literature on ochratoxin and their possible detrimental effects on poultry birds and subsequent public health hazards. Recent studies have revealed that embryos, new born chicks and young poultry are more sensitive to ochratoxin A than adults. Ochratoxin-A has a high affinity for liver, kidneys, bursa of Fabricius and thymus. It causes an appreciable increase in the size of liver and kidneys where as the size of bursa and thymus is reduced. It also causes nephrotoxicity and hepatotoxicity with carcinogenic effect. In embryo, it causes teratologic defects in the form of anophthalmia followed by mandibular hypoplasia, micropthalmia, maxillary retrognathism, reduced body size, everted viscera, spina bifida and exencephaly. Biochemically it causes hypoproteinemia, hypoalbuminemia, hypoglobulinemia and hypoglycaemia. Similarly, it also causes increased levels of blood urea nitrogen (BUN), serum creatinine, uric acid, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum triglycerides. In order to prevent and reduce implications of these mycotoxins in poultry feed, there is needs for both global and national strategic programs to reduce the residual accumulation of mycotoxins in grain, to use advanced analytic techniques and to establish new limits concerning the maximum amount of mycotoxins allowed in poultry feed and products from poultry for human consumptions.

Key words: Ochratoxin, toxicity, teratologic defects, immunoglobulins.

INTRODUCTION

Ochratoxins are the most common and dangerous mycotoxins in the poultry feed. The presence of ochratoxins in poultry feed leads to the development of health disorders in human beings and the decrease in production

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performance of poultry. This contributes to huge economic losses to the poultry industry due to increased mortality, reduced body weight gain, altered egg quality and egg production, increased feed conversion ratio, immunosuppression, early embryonic death and embryonic abnormalities. Residual accumulation of ochratoxins in meat and eggs is of public health concern because of consumption of ochratoxin-contaminated poultry products. Ochratoxins are a member of highly toxic compounds consisting of three members, A, B and C which are structurally related and are produced as secondary metabolites by several species of fungus. The name ochratoxin comes from *Aspergillus ocharaceous*. Ochratoxins are mostly produced by *Penicillium verrucosum* but five other species of *Aspergillus* and six other species of *Penicillium* produce it as well. So far, ochratoxin A (OTA) out of A, B and C is the most commonly detected and the most toxic member of the family. OTA is a common contaminant of cocoa beans, peanuts, soya and coffee in particular, the liver, kidneys and bursa of Fabricius are particularly affected by this toxin (Gibson et al., 1990). They are the second major group of mycotoxins to be characterized after the aflatoxins. Structurally, the three toxins differ only very slightly from each other; however, these differences have marked effects on their respective toxic potentials, with ochratoxin-A (OTA) being the most toxic (Peckham et al., 1971; Chang et al., 1979). Considerable species and sex differences in sensitivity towards OTA acute toxicity and half-life have been demonstrated (O'Brien and Dietrich, 2005). The *Aspergillus* OTA producers include strains of seven species in section Circumdati (*Aspergillus ochraceus*, *Aspergillus melleus*, *Aspergillus auricomus*, *Aspergillus ostianus*, *Aspergillus petrakii*, *Aspergillus sclerotiorum* and *Aspergillus sulphureus*), two species in section Flavi (*Aspergillus alliaceus* and *Aspergillus albertensis*), two species in section Nigri (*Aspergillus niger* and *Aspergillus carbonarius*), and one species in section Aspergillus (*Aspergillus glaucus*) (Bayman et al., 2002). Two Penicillium species, *Penicillium verrucosum* and *Penicillium nordicum*, share the ability to produce OTA (Larsen et al., 2001).

The natural occurrence of OTA in food and feedstuffs of plant and animal origin is very common. Due to its long half-life, OTA accumulates in the food chain, and threatens human and animal health because of its extreme toxicity, widespread occurrence and the variety of commodities that it can contaminate (Scott, 1978). OTA has been implycated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neuro-toxicity and immunotoxicity in both animals and man (O'Brien and Dietrich, 2005).

Effect of ochratoxin on body weight

Ochratoxin-A has a multifaceted effect on body weight of poultry. As the exposure to ochratoxin is increased, a decrease in feed consumption has been reported in broilers (Kumar et al., 2003) similarly, decrease in the body weight was reported by different workers in broilers and layers (Elaroussi et al., 2006; Hanif et al., 2008). Exposure of birds for long duration also causes reduced feed consumption. Two most important factors, that is, exposure level and exposure period are the most impor-tant conducive factors for a decrease in body weight. The reduction in feed consumption was more noticeable with time and with the higher level of OTA. Effect of OTA on cumulative feed conversion ratio was dose dependent (Elaroussi et al., 2006). The OTA responses studied in several studies were dose and time dependent. The decrease in broiler body weight due to ochratoxicosis was studied by several workers using dietary OTA inclusion rates of 567 ppb (Garcia et al., 2003), 0.5 to 2 parts/10^6 (Prior et al., 1980; Campbell et al., 1983; Kubena et al., 1988; Raju and Devegowda, 2000; Kumar et al., 2003), 1 to 4 parts/106 (Gibson et al., 1989; Verma et al., 2004), 5 parts/10^6 (Stoev et al., 2002) and up to 8 parts/10^6 (Huff et al., 1974, 1980, 1988).

Effect of ochratoxin on liver and kidneys

The effect of OTA on the liver and kidneys is more pronounced as both the liver and the kidney are involved in detoxification and elimination of OTA from the body. Enlargement in both organs on OTA feeding has been reported (Elaroussi et al., 2008). Increased relative weights of liver and kidneys were observed at lower dietary OTA levels when compared with those reported earlier (Elaroussi et al., 2008). This trend was inversely related with dietary OTA levels (Zahoor-ul-Hassan et al., 2011). The enlargement of both organs is probably due to enlargement of epithelium and increased hyperaemia or mononuclear cell infiltration in these organs. As OTA has high plasma protein binding ability due to which its elimination through glomerular filtration might be retarded. This toxin is excreted through kidney tubules using organic anion transporter proteins and is also reabsorbed in all nephron segments using organic anion transporter proteins or might be by other transporters. The reabsorption process reduces OTA excretion, leading to its accumulation in renal tissue and thus contributing to renal toxicity (Dahlmann et al., 1998; Pfohl-Leszkowicz and Manderville, 2007). Ochratoxin-A is also excreted through hepatobiliary route, enterohepatic circulation, and reabsorption in tubules might lead to degenerative changes and enlargement of epithelial cells of the liver and kidneys (Stoev et al., 2000). Gross enlargement of liver and kidney has also been reported by different workers (Kumar et al., 2004; Elaroussi et al., 2008). Similar findings on enlargement of liver and kidneys have been reported in layer chicks hatched from OTA inoculated eggs (Hassan et al., 2012). Pathological changes in the liver and kidney on feeding ochratoxin to broiler chickens have been reported earlier by Huff et al. (1974), Dwivedi and Burns (1984a), Kubena et al. (1985) and Mohiuddin et al. (1992).

Effect of ochratoxin on embryos

Ochratoxin A causes teratogenic effects in the embryos in the form of anopthalmia, mandibular hypoplasia, maxillary retrognathism, everted viscera, microphthalmia, spina bifida, exencephaly, and reduced body size by Gilani et al. (1978). These effects of OTA may be due to DNA adduct formation and subsequently inhibition of protein synthesis (Petkova-Bocharova et al., 2003). Embryonic mortalities in the OTA contaminated diet may be attributed to cytotoxic effects (Wei and Sulik, 1996; Choudhury and Carlson, 1973) and mice embryos (Wei and Sulik, 1993), intoxicated with different doses. No literature is available on the embryonic mortality induced by OTA in chicken embryos to the stage of development (Celik et al., 2000; Neldon-Ortiz and Qureshi, 1992). Morphometric studies of embryos shows that ochratoxin-A causes reduction in the size of embryos and this reduction is OTA dose dependent.

Effect of ochratoxin on lymphoid organs and biochemical parameters in poultry

Ochratoxin A causes a immunoglobulin levels to decrease in fowl (Dwivedi and Burns, 1984b) together with a regression of almost all the lymphoid organs (Peckham et al., 1971; Dwivedi and Burns, 1984a). OTA has also been shown to result in retarded growth and thymic regression in 3-week-old turkey and poultry (Chang et al., 1981). Study indicates that the effect of dietary ochratoxin on the histology of the bursa of Fabricius and thymus has shown necrosis and degeneration. The exposure of birds to 2 ppm ochratoxin-A, in the presence or absence of aluminosilicate, reduced their humoral immune response and the number of mitotic cells in the bursa and thymus. A decrease in the relative weight of thymus and bursa could be because of the necrotic and degenerative changes in these organs that results in the lower immune responses as described earlier (Stoev et al., 2000), Atrophy of the bursa or a decrease in its relative weight in broiler chicks fed ochratoxin A has been reported by Huff et al. (1974) and Kubena et al. (1985). The necrotic and degenerative changes in lymphoid organs (bursa of Fabricius and thymus) were similar as described earlier (Stoev et al., 2002; Elaroussi et al., 2006; Hanif et al., 2008). Ochratoxin A caused impaired immune function and perhaps explains the increased incidence of air-sacculitis in natural disease outbreaks of ochratoxicosis in turkeys (Hamilton et al., 1982). Creppy et al. (1979) suggested that the immune-suppressive effects of OTA might be due to an inhibition of protein and noted lymphocytopaenia and a significant depression in bursal weight and complement activity in fowls treated with both OTA and aflatoxin.

Ochratoxin A in the poultry diet causes alteration in hematologic parameters as reduction in RBC count, Hb concentration and PCV in broilers. Mohiuddin et al. (1993) who added OTA at concentrations of 0.75 - 3.0 mg/kg diet of broiler chicks similar to Stoev et al. (2000), who showed only a significant decrease of RBC count, a decrease in PCV and Hb concentration levels was reported, and attributed it to iron deficiency anemia or as a consequence of a disturbance in the haemopoietic system (Huff et al., 1988). Feed contaminated with OTA causes a significant decrease in WBC count of broilers (Chang et al., 1979; Mohiuddin et al., 1993). Leucocytopaenia was noted by Chang et al. (1979), for the highest dose of OTA. The decrease in the number of leucocytes was reported to be a reflection of a decrease primarily of lymphocytes, and to a lesser extent monocytes (Chang et al., 1979) or heterophils (Chang et al., 1981; Mohiuddin et al., 1993). Such a lymphocytopaenia may be a sensitive and useful indicator of ochratoxicosis that possibly occur due to a direct effect on germinal centers of lymphoid tissues and implies alteration of the immune function. The detrimental effects of OTA on WBC counts were also found in male turkey fed diets contaminated with (Chang et al., 1981), and in Japanese quail administered with OTA by (Farshid and Rajan, 1996). Therefore, O'Brien and Dietrich (2005) attributed the OTA-impaired immunity to a reduction in the proliferating lymphocytes, activation and differentiation of lymphocytes. OTA Ochratoxin-A and Citrinin have multi-faceted effects on biochemical parameters in poultry, it causes hypoproteinemia, hypoalbuminemia, hypoglobulinemia and hypoglycaemia. Similarly, it also causes increased levels of blood urea nitrogen (BUN), serum creatinine, uric acid, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum triglycerides in various studies (Jayaramu et al., 2012). Toxicopathological effects of feeding of OTA ochratoxin-A contaminated feed to broiler chicks for 21 days causes a decrease in the feed intake and body weight with behavioural alterations included diarrhea, depression, increased water intake and ruffled feathers. Synergistic effect of ochratoxin along with *Escherichia coli*-challenged broiler chickens causes increased serum levels of aspartate aminotransferase, alanine aminotransferase, uric acid and creatinine and decreased levels of total proteins, albumin, globulins, calcium, and phosphorus were observed in OTA-fed birds. The presence of OTA in poultry rations increased mortality and the severity of an *E. coli* infection (Kumar et al., 2004). Combinations of OTA and T-2 toxin causes significant decrease on immune function of broiler chickens changing the CD4+/CD3+ and CD4+/CD8+ ratios even at a concentrations as low as 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin (Wang et al., 2009).

Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 8(35), pp. 3220-3229, 27 August, 2014
DOI: 10.5897/AJMR2013.6465
Article Number: DEB066D47144
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Dental plaque of children as probable *Helicobacter pylori* reservoir

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Received 30 October, 2013; Accepted 7 July, 2014

Helicobacter pylori **is a spiral gram negative bacterium that normally grows in the stomach, and is the main causal agent of gastritis and peptic ulcers. In 1994, The International Agency for Research on Cancer recognized *H. pylori* as a carcinogenic factor for stomach cancer. The oral cavity has been proposed as a reservoir for *H. pylori* and several authors have demonstrated the presence in adult patients of the organism in saliva and dental plaque. However, isolation of *H. pylori* in children is still questionable. Several investigators have reported the presence of *H. pylori* in the dental plaque of children, sometimes associated with gastro-esophageal reflux. These isolations has been realized by polymerase chain reaction were gold standard is the culture. On the other hand, many authors have failed to isolate *H. pylori* by culture in samples obtained from the saliva or dental plaque. We performed an extensive review of the literature to facilitate future research in this controversial topic.**

Key words: *Helicobacter pylori*, children, dental plaque, polymerase chain reaction, urease breathe test.

INTRODUCTION

Since the discovery of the presence of *Helicobacter pylori* in the stomach, there have been many studies trying to determine the source of colonization of this bacterium (Dunne et al., 2014). The oral cavity has been proposed as a reservoir for *H. pylori* by several authors that demonstrated the presence of the organism in dental plaque and saliva from adult patients (Sudhakar et al., 2008; Agarwal and Jithendra 2012; Liu et al., 2013).

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However, in children many researchers have failed to isolate the bacterium from these sites (Muñoz et al., 1999; Silva et al., 2009). This might be due to intermittent presence of the bacterium in dental plaque or due to its lesser number (Song et al., 2000). The isolation of *H. pylori* in children is still questionable, though there are studies that have associated gastro-esophageal reflux with the presence of *H. pylori* in children's oral cavity (Emiroglu et al., 2010). On the other hand, most of the studies where isolation of H*. pylori* is reported come from polymerase chain reaction laboratory test, but the standard for Helicobacter isolation is the culture (Koido et al., 2008). The main objective of this review was to verify if the hypothesis that dental plaque serves as a reservoir for *H. pylori* can be justified from the literature.

Helicobacter pylori

More than three decades ago it was thought that the human stomach was free of bacteria because of its very acid pH (Macy et al., 1978, Drasar et al., 1969). But by 1984, Dr. Robin Warren and his colleague Dr. Barry Marshall described helical-shaped bacteria that could survive and colonize the gastric chamber (Marshall and Warren, 1984). *H. pylori* is a Gram negative spiral or curve-shaped rod that normally colonizes the stomach (Owen, 1998).

Epidemiology

H. pylori infection is one of the most common infections in the world. Its prevalence varies according to geographical area. Developing and oriental countries have major rates of disease than developed and industrialized countries (Pounder and Ng, 1995). Lower socio-economic factors are related to mayor prevalence of infection (Pounder and Ng, 1995). Most of the reports on the prevalence of *H. pylori* infection come from serum-epidemiologic studies (Parsonnet, 1995). *H. pylori* is thought to be indigenous to the human population and is well adapted to the harsh conditions of the human stomach, which is colonized for the host's lifetime (Blaser, 1997). Although the principal reservoir for *H. pylori* infection appears to be humans, *H. pylori* has been isolated from non-human primates and domestic cats (Dubois et al., 1996; Handt et al., 1994). Infection is generally asymptomatic (Blaser, 1995), but may develop to a chronic gastritis (Sipponen, 1997). *H. pylori* has been recognized as a major cause of gastritis and is associated with duodenal ulcer disease, gastric ulcer disease, gastric lymphoma, and gastric cancer in humans (Konturek et al., 2006; Konturek et al., 2009; Kusters et al., 2006; Ando et al., 2006; Ito et al., 2006; Kato and Asaka, 2012). These complications of infection also display geographic preference, which may be due to variations in the strain and virulence factors of *H. pylori* (Suzuki et al., 2012). Besides, complications such as atherosclerosis with acute heart stroke and lymphomas have been associated with *Helicobacter* infections (Kinoshita, 2007; Witkowska and Smolewski, 2013).

Helicobacter infection displays no preference for the hosts gender (Kim, 2005), but its incidence increases between with increasing age of the host (2 to 20 years) and then remain stable (Kim, 2005).

Diagnosis

The urea breath test (UBT) is a very simple, innocuous and highly accurate test strongly associated to *H. pylori* infection (Bytzer et al., 2011). UBT is particularly suitable in all clinical conditions where endoscopy is not strictly necessary, and to check the success of eradication regimens (Atherton and Spiller, 1994; Logan, 1998; Savarino et al., 1999). Another frequently used test for the diagnosis of Helicobacter infection is the rapid urease test (RUT) in gastric biopsy, which is viable in commercial rapid probes (Marshall et al., 1987; Hazell et al., 1987). The antigenic determination of *H. pylori* in feces has been approved by the US Food and Drug Administration for detection and follow-up testing (Monteiro et al., 2001). Fecal antigen detection and UBT are recommended non-invasive approaches for confirmation of infection in children especially those with less than 5 years age. Recently, more sensitive and specific molecular diagnostic tests have been developed. Strategies for polymerase chain reaction (PCR)-based detection of *H. pylori* have included multiple genetic targets with varying levels of sensitivity and diagnostic accuracy. (Lu et al., 1999; Maeda et al., 1998; Gramley et al., 1999). Detection of virulence genes has been offered the best results. However culturing *H. pylori* remains the gold standard for detection of Helicobacter (Koido et al., 2008).

Serologic testing represents a primary screening approach for evaluation of *H. pylori* status in patients not immediately requiring endoscopic studies. With respect to enzyme-linked immunoassays, serum samples yield higher sensitivity and accuracy as compared to whole blood samples (European *Helicobacter pylori* Study Group, 1997; Faigel et al., 2000).

Reservoir

Since the isolation of bacteria from the stomach, researchers have searched the source of Helicobacter, including food, water and the periodontal plaque, as a reservoir from which *H. pylori* is ingested to reach the stomach and thus colonize it.

The presence of *H. pylori* in the oral cavity is still controversial as many studies claimed *H. pylori* presences based on the UBT or immunological tests without confirmation by culture or molecular assays (Al Asqah et al., 2009; Jia et

al., 2009; Koumi et al., 2011). Definitely the only way to reliably demonstrate the presence of this bacterium is its culturing, which requires special and complicated conditions, still, successful *H. pylori* cultures obtained from oral samples of 16/20 patients have been reported (D'Alessandro and Seri, 1992).

Variations in the detection of *H. pylori* by molecular techniques can be explained by differences in: 1) sampling sites (saliva, periodontal pockets, dental plaque) (Fritscher et al., 2004; Liu et al., 2008; Medina et al., (2010), Tsami et al.,2011); 2) sample handling (DNA extraction from isolation sites directly or after culturing) (Anand et al., 2014); 3) primers and geographical distribution of strains (Göttke et al., 2000), and 4) oral health status of the patient (healthy, multiple cavities, mouth ulcers, periodontal disease) (Brown, 2000). Table 1 provides a summary of results of molecular detection from oral samples from adults.

On the other hand, Table 2 summarizes the conditions related with negative results of molecular detection. The success rate in isolating *H. pylori* from the dental plaque of infected subjects varies between 0 and 88% (Pustorino et al., 1996; Pytko-Polonczyk et al., 1996; Majmudar et al., 1990; Desai et al., 1991; Nguyen et al., 1993; Bernander et al., 1993; Bickley et al., 1993; Asikainen et al., 1994; Hardo et al., 1995). A recent systemic review of literatures seems to indicate that dental plaque may be a potential reservoir for *H pylori* according to recent systematic reviews (Anand et al., 2014).

Reservoir in children

Once it was accepted that the oral cavity could serve as a reservoir for *H. Pilory*; the question arise when, during human life, the oral cavity becomes colonized. Studies in Mongolian gerbils, has demonstrated that vertical transmission occurs in the first 4 months of life (Oshio et al., 2009) while Lee and et al. (2006) could not demonstrate the vertical transmission from mother to newborns in the same murine model.

Studies in newborn and mothers from maternal child care hospital in Italy demonstrated than 34.8% of the mothers and 2.9% of the newborns have stool antigen test (SAT) positive to *H. pylori* infection (Baldassarre et al., 2009).

However same author consider that SAT is not a good test to demonstrate the vertical transmission of Helicobacter (Baldassarre et al., 2008).

Another author has demonstrated by PCR that 46% of the mothers infected by *H. pylori* have related DNAstrains that her children. However, the vertical transmisión of these strains is not sure (Nahar et al., 2009).

Actually, the culture isolation and PCR detection has not been demonstrated in the newborn. Although, most of the studies point to maternal child transmission (Weyermann et al., 2006), it causal relation has not been demonstrated. Thus, another source of infection should be considered, and then water, food (Vale and Vítor, 2010), animals (Brown, 2000) and siblings (Schwarz et al., 2008) were associated as sources of infection of *H. pylori*.

The main concern emerge in the possibility of dental plaque as source of infection or re-infection since primary source may be mother siblings, water, food or animals; mouth is an obligatory way to transit to the gastric cavity (Brown, 2000). *H. pylori* grows better in a micro-aerophilic environmental conditions; these are the conditions of dental plaque neighborhood (Atherton, 2006). The main hypothesis is that dental plaque is colonized in early stages of the life. Then it works as reservoir, but this hypothesis cannot be completely recognized. The main obstacles to demonstrate it, merges from studies that suggest that the dental plaque colonization is caused by gastro-esophageal reflux, and not in the reverse way.

Other obstacles arise as the detection by molecular identification suffers from the same variety of outcomes as described for adults. However, the isolation of *H. pylori* in the dental plaque of children remains as a controversial topic. There are researchers who reported isolations of *H. pylori* in dental plaque (Tsami et al., 2011; Chaudhry et al., 2011; Gill et al., 1994; Liu et al., 2008; Ou et al., 2013; Valdez-Gonzalez et al., 2014), but others have been unable to detect *H. pylori* in the oral cavity (Olivier et al., 2006; Bernander et al., 1993). The study conditions that have allowed for identification of *H. pylori* for children's oral cavities or not are summarized in Tables 3 and 4 respectively (Muñoz et al., 1999; Santamaria et al., 1999; Kignel et al., 2005).

Several explanations for discordant results are: a) inadequate and poorly designed primers for microbial detection, b) inadequate sampling from patients and sample processing, c) small patient numbers (Olivier et al., 2006), and d) intermittent or and/or scarce presence of the *H. pylori* in the oral cavity (Song et al., 2000).

In developing countries, children are infected at an early age, usually before the age of two. The suggested routes of transmission are fecal-oral, oral-oral, gastric-oral (Sahay and Axon, 1996; Tursi et al., 1997); all related to poor hygienic conditions. Also vertical transmission, from mothers to baby, can happen, as well as horizontal transmission of microorganism from parents or other caregivers to children (Kitagawa et al., 2001).

The concomitant presence of gastro-esophageal reflux and *H. pylori* detection in dental plaque suggests transmission of the bacteria from the stomach to the mouth and not in the other way and arguments against the hypothesis that the dental plaque may serve as a reservoir for gastric colonization (Emiroglu et al., 2010).

Findings of *H. pylori* in children with gastritis and gastrointestinal symptoms are common (Ogunbodede et al., 2002; Medina et al., 2010). Furthermore, *H. pylori* has been detected in the dental plaque of otherwise healthy adults (Tsami et al., 2011).

Finally the virulence expression factors of *H. Pylori* in

Table 1. Authors who has reported positive isolation of *H. pylori* in adults.

Author	Patients	Isolation (%)	Method
Majmudar et al. (1990)	40	100	CLOtest, culture and smear
Desai et al. (1991)	43	98	CLOtest
D'Alessandro et al. (1992)	20	80	Culture
		100	Urease
Malaty et al. (1992)	239	24	IgG antibodies
Nguyen et al. (1993)	25	38.8	RT-PCR immunofluorescence
Mapstone et al. (1993)	30	5-17	PCR
Song et al. (1994)	40		Rapid urease test, anti-Hp fluorescein-labelled antibody staining, bacterial culture and electronic microscopy.
Cellini et al. (1995)	31	64	Urease test
		3.2	Culture
Zhou and Yang (1995)	3519	61.2	PCR
Yang (1993)	29	72.4	PCR
Herdo et al. (1995)	62	54	PCR
		8	Culture
Luzza et al. (1995)	152	86	IgG antibodies
Wallfors et al. (1995)	110	48	PCR
Cammarota et al. (1996)	31	3.2	PCR
Pustorino et al. (1996)	63	6	Culture
Peach et al. (1997)	217	30.6	IgG antibodies
Contractor et al. (1998)	100	81	Rapid urease test
		13	Rapid urease test
Oshowo et al. (1998)	208	7	PCR
		1	Culture
Amendula et al. (1998)	20	5	Culture, PCR
Mattama et al. (1998)	62	1.6	Culture
Kamat et al. (1998)	248	4	PCR
Riggio et al. (1999)	73	33	PCR
Song et al. (1999)	40	27-100	PCR
Dore et al. (1999)	24	40.9	PCR
Huw et al. (1999)	13	84.6	PCR
Butt et al. (1999)	173	100	CLOtest
Miyabayashi et al. (2000)	47	25.5	PCR
Song et al. (2000)	42	97	PCR
Kim et al. (2001)	46	6.9-28.6	PCR
Younj et al. (2001)	5	100	Electronic microscopy
Avcu et al. (2001)	108	28.5-100	Camphylobacter-like organism test gels
Honda et al. (2001)	60	42-70	IgG ELISA
Kitagawa et al. (2001)	1588	29.2	IgG antibodies and PCR
Goosem et al. (2002)	58	3	PCR
Al-Refai et al. (2002)	135	89	Urease
Mazuda et al. (2002)	116	42.3	IgG antibodies
	116	40	
Ozdemir et al. (2001)	81	79	Urease
Butt et al. (2002)	78	100	CLOtest
		88	Citology
Huw et al. (2002)	32	84	PCR
Berroteran et al. (2002)	32	37.5	PCR
Suk et al. (2002)	65	43	PCR

Table 1. Contd.

Umeda (2003)	57	35.1	PCR
Gürbüz et al. (2003)	75	90	CLOtest
Ogunbodede et al. (2002)	66	69.7	Culture and biopsy
Siddig et al. (2004)	52	92.3	Urease test
Al-Hawajri et al. (2004)	24	50	PCR
Cześnikiewicz et al. (2004)	100	48.3	Culture
Kignel et al. (2005)	49	2	PCR
Anand et al. (2006)	134	71-89	Rapid urease test and serology
Gebara et al. (2006)	30	46	PCR
Chitsazi et al. (2006)	88	34	Rapid urease test
De Souza et al. (2006)	97	99.3	Rapid urease test
Loster et al. (2006)	40	48	Culture and PCR
Chumpitaz et al. (2006)	115	3.5	Culture
Teoman et al. (2007)	67	28.3	PCR
Liu et al. (2008)	126	55	PCR
Bürgers et al. (2008)	94	17	PCR
Sudhakar et al. (2008)	15	11.9	Culture and RUT
Liu et al. (2009)	443	59.4	PCR
Al Asqah et al. (2009)	101	65	Rapid urease test
Silva et al. (2009)	62	36.6	PCR
Jia et al. (2009)	148	19.1	Rapid urease test
Medina et al. (2010)	98	18	PCR
Eskandari et al. (2010)	67	5.97	PCR
Leszczyńska et al. (2009)	164	81.2	Immunoassay
Trevizani et al. (2010)	78	47.4	PCR
Chaudhry et al. (2011)	150	37.5	PCR
Assumpção et al. (2010)	71	89	PCR
Fernández et al. (2011)	200	17	PCR
Silva et al. (2010)[a]	30	20	PCR
Silva et al. (2010)[b]	115	25	PCR
Momtaz et al. (2010)	250	14.4	PCR
Koumi et al. (2011)	56	41.5	Rapid urease test
Diouf et al. (2011)	109	14.7	PCR
Momtaz et al. (2012)	300	77.6	PCR
Agarwal et al. (2012)	30	60	PCR and culture
Liu et al. (2013)	574	68.2	

dental plaque suggest that the bacteria express active virulence factors while growing in the dental plaque. This finding plus major expression of factors and major bacterial counts in the periodontal disease suggest that dental plaque works not only as reservoir by contamination but it really infect and cause damage in the periodontal tissue (Tsami et al., 2011).

There are studies that have shown that strains that are present in the dental plaque and in the stomach are different. In this way, the controversy continue because enough information does not exist that demonstrate transmission from the mouth to the chamber gastric or chamber gastric-dental plaque (Cai et al., 2014).

Other important issue emerges from the fact that *H. pylori* isolation in the most studies is realized by PCR were culture is the gold standard for the diagnosis.

PERSPECTIVE

Recent literature seems to favor the view that the dental plaque may serve as a reservoir for *H. pylori*, both in healthy adults and patients suffering from oral disease, especially periodontal disease (Anand, 2014).

Table 2. Authors who has been reported negative isolation of H. pylori in adults.

Author	Patients	Method
Dalen et al. (1993)	94	Culture
Bickley et al. (1993)	15	Culture
Von Recklinghausen et al. (1994)	49	Culture
Asikainen et al. (1994)	336	PCR
Luman et al. (1996)	1020	Culture
Cheng et al. (1996)	244	Culture
Savoldi et al. (1998)	80	Immunoperoxidase test
Muñoz et al. (1999)	53	PCR and culture
Birek et al. (1999)		PCR
Sahin et al. (2001)	23	PCR-RFLP
Olivier et al. (2006)	79	Histology and PCR
Silva et al. (2009)	62	PCR

Table 3. Authors who has been reported positive isolation of *H. pylori* in children.

Author	Patients	Isolation (%)	Method
Gill et al. (1994)	22	82	Rapid urease test
Muñoz et al. (1999)	53	84.3	PCR and culture
Qureshi et al. (1999)	100	28.3-40	IgG antibodies
Song et al. (2000)	6	100	PCR
Patty et al. (2002)	140	7.8	PCR
Allaker et al. (2002)	15	68	PCR
	22	0	Culture
Ogunbodede et al. (2002)	12	69	Culture and histology
Fritscher et al. (2004)	105	5.7	PCR
Liu et al. (2008)	240	51	PCR
Medina et al. (2010)	98	18	PCR
Wichelhaus et al. (2011)	11	82	PCR
Tsami et al. (2011)	35	42.8	PCR
Hirsch et al. (2012)	3	66	PCR
Ou et al. (2013)	138	21.7	PCR

The identification of *H. pylori* by culturing or PCR was quite consistent. The design of a variety primers and probes enables to detect several strains types according to their geographical distribution and even though they are present in small quantities (Diouf et al., 2011; Song et al., 2000). The successful identification of *H. pylori* in the oral cavity is mostly achieved from dental plaque samples, and seems to be more difficult from saliva samples (Song et al., 2000).

The microaerophilic conditions of the periodontal pockets appear to favor the growth of bacteria, while saliva may serve as a transient vehicle. In adult patients, the flaws in the isolation may be related to the sample site; as we have already mentioned saliva samples may be inadequate (Madinier et al., 1997).

Appropriated culture conditions are essential for *in vitro* growth of the bacteria. Failure to culture bacteria may be due to lack of experience in handling these bacteria, such as small variations in the culture medium, technique or incubation conditions (Ndip et al., 2003). Similarly, the failure to detect *H. pylori* by PCR may be due to methodological flaws from the site of the fetched sample, the sample size, the primers used, the reagents used and failures in amplification protocols (Al Sayed et al., 2014). The isolation of *H. pylori* from children is not exempted from the problems associated with isolation in adults.

The concomitant presence of gastro-esophageal reflux and *H. pylori* identification in the oral cavity is an

Table 4. Authors who has been reported negative isolation of *H. pylori* in children.

Author	Patients	Method
Santamaría et al. (1999)	53	PCR
Oliver et al. (2005)	79	PCR and culture
Song et al. (2000)	6	PCR
Allaker et al. (2002)	22	Culture
Hirsch et al. (2012)	3	Culture

argument against the hypothesis that the dental plaque serves a reservoir. Indeed, the presence of *H. pylori* in the periodontal pockets may be a contamination of the normal oral microflora by gastric reflux (Kurtaran et al., 2008).

It is important to determine the infection moment as well as to establish the route of transmission. Another problem is to demonstrate the presence of *H. pylori* in Edendule adult patients (Cheng et al., 1996), but it does not happen in kids (Alarcón et al., 2013; Mourad-Baars et al., 2010).

In conclusion, although *H. pylori* has been identified in the oral cavity of children, additional studies are needed to support the hypothesis that the dental plaque serves as a reservoir of *H. pylori* in children.

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Vol. 8(35), pp. 3230-3234, 27 August, 2014
DOI: 10.5897/AJMR2014.7006
Article Number: 4E540F347146
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

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Evaluation of fungicide application on late blight in popular potato cultivars of the north eastern Himalayan hills of India

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Received 10 July, 2014; Accepted 22 August, 2014

Potato late blight is one of the major constraints to potato cultivation in the north eastern hill region of India. Eight popular potato cultivars (Kufri Girdhari, Kufri Megha, Kufri Himsona, Kufri Himalini, Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja) were evaluated for late blight disease progress and yield potential with or without the application of fungicide in the field. Blight appeared 103 days after planting (DAP) in Kufri Girdhari and 89 DAP in Kufri Megha in sprayed and unsprayed plots. Minimum terminal disease severity was recorded in Kufri Girdhari (6.5 and 7.5%) followed by Kufri Megha (22.5 and 35%), Kufri Himsona (62.5 and 70%) and Kufri Himalini (70 and 75%) in sprayed and unsprayed plots, respectively. Area under the disease progress curve was lowest on Kufri Girdhari (22.75 and 26.25) followed by Kufri Megha (253.75 and 402.5), Kufri Himalini (934.5 and 1333.5), Kufri Himsona (813.75 and 1162), Kufri Giriraj (1347.5 and 2082.5), Kufri Jyoti (1382.5 and 2117.5), Kufri Kanchan (1277.5 and 1951.25) and Kufri Shailja (1333.5 and 2073.75) in sprayed and unsprayed plots, respectively. There was no significant difference in areas under disease progress curves (AUDPCs) and yield of sprayed and unsprayed plots of Kufri Girdhari, whereas, significant differences (cultivar and spray) were recorded in Kufri Megha, Kufri Himalini and Kufri Himsona. The highest tuber yield was obtained in Kufri Girdhari (34.08 t/ha) and Kufri Himalini (33.48 t/ha). The study revealed that Kufri Girdhari gave highest yield with and without fungicidal applications.

Key words: Late blight, *Phytophthora infestans*, *Solanum tuberosum* disease severity, fungicidal spray, cultivars, yield.

INTRODUCTION

Potato is one of the most important vegetable crops grown in the North Eastern Hill (NEH) region of India, which is comprised of the states of Arunachal Pradesh, Mizoram, Nagaland, Manipur, Meghalaya, Tripura, Sikkim and Assam, and accounts for nearly 10% of the country's total potato area. The potato yield in all North

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Figure 1. Photograph showing symptoms of potato late blight of Kufri Jyoti cultivar in sprayed plots.

Eastern states except Tripura (17.8 t/ha) is fairly low (4.8 to 12.6 t/ha) as compared to the national average of 22.24 t/ha (NHB, 2012). One of the major constraints in attaining good yield is late blight disease in the mid- to high-hills. The entire NEH region is characterised by undulating topography, rain-fed cultivation and inadequate management practices (Sah et al., 2007). Late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is one of the most important diseases of potato. It occurs in epiphytotic proportions every year in the NEH region where, under favorable cool and moist conditions, the pathogen can cause considerable loss to the crop, making it almost impossible to achieve good yield in conventional potato production without the use of fungicides. In India, late blight appears in most of the potato growing regions in varying degrees, causing yield losses of up to 90% (Singh et al., 2003).

In general, late blight disease is managed by cultural practices, host resistance and the use of fungicides. However, the development of pathogen resistance to fungicides and the evolution of new strains of the pathogen have compounded the situation (Singh, 1996). The use of resistant cultivars is considered to be the most effective and environment-friendly strategy to manage late blight but commercially important characteristics, such as quality, yield, earliness, etc., are usually not combined with late blight resistance in the same cultivar (Cooke et al., 2011). Currently, the disease is generally managed by spraying fungicides at regular intervals, which adds to the cost of production and poses serious environmental hazards. In the NEH region of India, few resistant cultivars are available for cultivation. Also, new fungicides are rarely available in the NEH region and farmers often are reluctant to use them because of the cost involved. Late blight disease can be managed economically by planting resistant cultivars coupled with judicious, need-based application of effective fungicides. Therefore, the present study was undertaken to analyze the progress of late blight disease in popular potato cultivars recommended or being cultivated in the Meghalaya hills with and without the application of fungicides.

MATERIALS AND METHODS

The experiments were conducted at the Central Potato Research Station, Upper Shillong, Meghalaya, India (1800 m amsl, 25.54°N, 91.85°E) in the summer seasons (February-July) of 2012 and 2013 under rain fed conditions. Eight cultivars, namely Kufri Girdhari, Kufri Giriraj, Kufri Jyoti, Kufri Himalini, Kufri Himsona, Kufri Kanchan, Kufri Megha and Kufri Shailja, were planted on 25 February in both years in a randomized block design with three replications, each replication constituting of six rows of 2 m each at spacing of 60 cm between rows and 20 cm between plants (ridge and furrow method). The totally disease free seed potato (40-60 g) were taken for planting. Sixty whole seed potato tuber were planted in each plot. The potato crop was raised as per the standard recommended package of practices. Under protected conditions, a fungicide spray schedule comprising of 1st spray with mancozeb @ 0.2% at time of canopy closure or before disease appearance; 2nd spray with cymoxanil + mancozeb@ 0.3% and 3rd spray with mancozeb @ 0.2% at 7 days interval was followed whereas no fungicide was sprayed under unprotected condition. Disease severity was recorded based on percent foliage infection at seven days intervals after first appearance of late blight (Malcolmson, 1976). The area under disease progress curve (AUDPC) was calculated according to Shaner and Finney (1977) as follows:

$$AUDPC = \sum_{i=1}^{n} [(x_i + x_{i+1})/2](t_{i+1} - t_i)$$

Where, n = total number of observations; x_i = disease severity at the i^{th} observation; I = time (days) at i^{th} observations.

Data on late blight severity was used to compute area under disease progress curves (AUDPC). The average late blight severity at weekly interval, AUDPC and tuber yield (t/ha) were subjected to two factor analysis of variance (ANOVA) in order to determine effects of cutivar and fungicide applications on disease progression.

RESULTS AND DISCUSSION

Late blight disease (Figure 1) appeared 75 to 82 DAP (3rd to 4th week of May) in both years (Table 1). In hills of Meghalaya, the peak growth period of potato coincides with monsoon rains and mild temperature; resulting in the regular occurrence of late blight in epiphytotic proportions (Srivastava et al., 2012). Late blight disease appeared (1-5% blight severity) in Kufri Himalini, Kufri Himsona, Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja at 75 DAP in the unsprayed crop, whereas, it was delayed by more than a week (15 DAP) in Kufri

Table 1. Average blight severity, AUDPC and tuber yield (t/ha) of potato cultivars under sprayed and un-sprayed conditions (pooled data of 2012 and 2013).

Cultivars	Crop age at first appearance of disease (days)	Average late blight severity (%) at 7 days interval starting at 75 DAP					AUDPC**	Tuber yield (t/ha)
		75	82	89	96	103 (TDS)*		
Unsprayed crop								
Kufri Girdhari	103	0	0	0	0	7.5	26.25	33.43
Kufri Megha	89	0	0	10	30	35	402.50	20.20
Kufri Himalini	75	1	25	57.5	70	75	1333.50	27.62
Kufri Himsona	75	1	15	50.5	65	70	1162.00	23.31
Kufri Giriraj	75	5	50	95	100	100	2082.50	22.75
Kufri Jyoti	75	5	55	95	100	100	2117.50	20.22
Kufri Kanchan	75	2.5	42.5	85	100	100	1951.25	18.91
Kufri Shailja	75	2.5	50	95	100	100	2073.75	17.15
Sprayed crop								
Kufri Girdhari	103	0	0	0	0	6.5	22.75	34.08
Kufri Megha	89	0	0	7.5	17.5	22.5	253.75	22.80
Kufri Himalini	82	0	1	42.5	55	70	934.50	33.48
Kufri Himsona	89	0	0	35	50	62.5	813.75	28.49
Kufri Giriraj	82	0	5	57.5	80	100	1347.50	27.97
Kufri Jyoti	82	0	5	60	82.5	100	1382.50	28.64
Kufri Kanchan	82	0	5	50	77.5	100	1277.50	22.98
Kufri Shailja	82	0	5	55	80.5	100	1333.50	20.79
CD (0.05)								
Cultivars		0.43	4.57	3.29	3.67	1.29	47.58	0.91
Spray		0.21	2.29	1.64	1.83	0.64	23.79	0.45
Cultivar x spray		0.61	6.47	4.65	5.19	1.82	67.29	1.29

*TDS = Terminal disease severity; **AUDPC = area under the disease progress curve.

Himsona and by less than a week in the other cultivars under fungicide protection. Based on AUDPC, Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja would be considered highly susceptible to late blight and Kufri Himsona and Kufri Himalini would be considered moderately susceptible. As compared to the susceptible cultivars, the appearance of late blight in Kufri Megha was delayed by about one week in the sprayed plots and about two weeks in the unsprayed plots, and the appearance of late blight in Kufri Girdhari was delayed by about three weeks in the sprayed plots and about four weeks in the unsprayed plots. These results obtained indicate that the weekly application of fungicide resulted in a reduction of late blight progression (Table 1). Earlier workers studied that Singh et al. (2001) have reported that late blight can be managed effectively even on susceptible cultivars by a timely fungicidal spray schedule.

The percent rate of disease progression was highest in Kufri Jyoti, Kufri Giriraj, Kufri Kanchan, and Kufri Shailja in unsprayed as well as in sprayed conditions but lowest in Kufri Girdhari, Kufri Megha, Kufri Himsona and Kufri Himalini. Minimum terminal late blight severity (at five weeks after appearance of disease and 103 DAP) was recorded in Kufri Girdhari (6.5%) followed by Kufri Megha (22.5 %), Kufri Himsona (62.5 %) and Kufri Himalini (70 %) in sprayed crop while it was hundred percent on Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja under both conditions. Prevailing weather conditions are well known factors that influence the disease spread and development of disease on the foliage.

Area under the disease progress curve (AUDPC) was highest on Kufri Jyoti (2117.5) in unsprayed crop and lowest on Kufri Girdhari (22.75). AUDPC of sprayed crop was 253.75, 934.5, 813.75, 1347.5, 1382.5, 1277.5, 1333.5 in Kufri Megha, Kufri Himalini, Kufri Himsona, Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja, respectively (Table 1) whereas overall AUDPC was highest in unsprayed as compared to sprayed crop. There were no significant differences in AUDPC and yield of Kufri Girdhari in sprayed and unsprayed but significant differences (cultivar and spray) were observed in terms of yield and AUDPC of Kufri Megha, Kufri Himalini, Kufri Himsona, Kufri Giriraj, Kufri Jyoti,

Table 2. Analysis of variance of the effect of fungicide sprayed and unsprayed on blight disease severity at weekly interval, AUDPC and yield (t/ha).

Source	d.f.	Mean sum of square due to						
		Disease Severity					AUDPC	Yield (t/ha)
		75 DAP	82 DAP	89 DAP	96 DAP	TDS# (103 DAP)		
Rep.	2	0.28	35.25	20.54	25.25	2.51	3005.25	1.26
Cultivars.	7	7.20**	1285.96**	6061.65**	7395.63**	7812.99**	2716907.81**	148.73**
Spray	1	70.33**	10785.01**	6063.76**	2133.33**	126.75**	2684038.55**	238.16**
Cultivar x spray	7	7.20**	758.89**	381.31**	49.79**	33.00**	127632.42**	8.04**
Error	32	0.13	15.04	7.78	9.68	1.20	1628.85	0.60

#Terminal disease severity; **significant at P = 0.01.

Kufri Kanchan and Kufri Shailja (Table 1).

Crop receiving alternative spray of mancozeb @ 0.2% followed by cymoxanil + mancozeb @ 0.3% and spray of mancozeb @ 0.2% responded significantly showing reduction in disease progress and increased yield under fungicidal protection. The highest total tuber yield was recorded in Kufri Girdhari (34.08 t/ha) followed by Kufri Himalini (33.48 t/ha) and Kufri Himsona (28.49 t/ha), in sprayed crop whereas Kufri Girdhari also gave highest yield (33.43 t/ha) significantly in unsprayed crop which indicated that fungicidal sprays have no impact on yield of Kufri Girdhari (Table 1). The tuber yield of 22.80 t/ha significantly obtained in Kufri Megha under protection. In the context of cultivar and spray interaction, tuber yield was significantly higher in Kufri Himalini followed by Kufri Himsona, Kufri Megha, Kufri Giriraj, Kufri Jyoti, Kufri Kanchan and Kufri Shailja. Although, terminal disease severity was hundred percent in Kufri Jyoti and Kufri Giriraj, both cultivars gave significantly higher yield (28.64 and 27.97 t/ha) under fungicidal protection. It may be due to short duration, varietal character and early bulking in addition to fungicide effect. Kufri Himalini, Kufri Himsona, Kufri Jyoti and Kufri Giriraj can also be grown with an effective spray schedule of three fungicidal applications. Overall, the results of the combined analysis of variance showed that the interaction of cultivars and spray was significant at P=0.01 (Table 2). Integrated approaches of late blight monitoring and effective fungicide application, resulted in significant increase in yield as compared to unsprayed crop. Still, farmers are growing local unhealthy seed and susceptible potato cultivars in the north eastern Himalayan hill region of India. Therefore, there are definite need of fungicide spray and stable high yielding resistant cultivars.

Conclusions

Late blight causes heavy yield losses in summer and autumn crops under rain fed conditions in the mid to high hills of Meghalaya. The study revealed that Kufri Girdhari gave highest yield without any fungicidal applications. Farmers can grow Kufri Girdhari without spray till the high degree of resistance persists. They can also grow cultivars *viz*. Kufri Himalini, Kufri Himsona, Kufri Jyoti and Kufri Giriraj successfully when accompanied by effective first spray of mancozeb @ 0.2% at the time of canopy closure or before the appearance of late blight, second spray of systemic fungicide (cymoxanil + mancozeb) @ 0.3% and third spray of mancozeb @ 0.2% fungicides.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Central Potato Research Institute, Shimla (H.P.) and the Head, Central Potato Research Station, Shillong for providing facilities during the study.

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academicJournals

Vol. 8(35), pp. 3235-3242, 27 August, 2014
DOI: 10.5897/AJMR2014.6897
Article Number: F021E7947148
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological and parasitic quality of *suya* (roasted beef) sold in Makurdi, Benue State, Nigeria

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Received 13 May, 2014; Accepted 8 August, 2014

This work is an investigation of the contaminants (microbial and parasitic) of *suya* (n=240) sold within Makurdi metropolis. The followings were detected in the roasted beef: bacteria: *Escherichia. coli, Staphylococcus aureus, Salmonella* sp., *Klebsiella* sp., *Shigella* sp., *Enterococcus* sp., *Streptococcus* sp., *Bacillus* sp., and *Pseudomonas* sp.; fungi: *Aspergillus* sp., *Penicillum* sp., *Rhizopus* sp. and y*easts;* protozoa: *Ascaris lumbricoides, Entamoeba histolyica,* Hookworm, *Taenia* sp. and *Giardia lamblia.* The moisture content of the meat increased when stored between 32.00 and 37.80%. The highest values of aerobic colony and coliform counts were 7.27 and 4.32 Log_{10}CFU/g respectively. Mean fungal and parasite counts ranged from 2.05 to 4.35 and from not detectable to 2.85 Log_{10}CFU/g respectively. Using a 95% level of significance, the concentration of the pathogen among the suya samples from the different markets were statistically different ($p<0.05$). To reduce the number of these pathogenic microorganisms and parasitic contents of suya, it is recommended to apply either hazard analysis and critical control points (HACCP) or International Organization for Standardization (ISO) 22000 along with prerequisite programs.

Key words: Ready-to-eat *suya*, microbial quality, bacteria, fungi, protozoa.

INTRODUCTION

Suya is a roasted or smoked beef or other boneless animal meat. Meat is rich in protein, zinc, vitamin B_{12} (cobalamin), selenium and phosphorus and is also a good source of niacin, vitamin B_6 (pyridoxine) and iron (Kramiliah et al., 1973). Suya is one of the most street-vended meat products in Nigeria and sub-Saharan Africa. Foods sold in streets can be easily contaminated by microbial and parasites due to the nature of the sellers and the environments.

Suya is prepared from boneless meat of animals (Abdullahi et al., 2004), mostly from lean meat. The meat can be roasted, smoked or dried, to increase its palatability and shelf life. The preparation processes involve defatting and slicing the meat on a slab or tabletop, after which it is staked into sticks, spiced and roasted for about 20 min. Thereafter, the product is spiced again and briefly reheated for about 2 min. It is then displayed for marketing on tabletops

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or trays where it can be street-vended (Edema et al., 2008).

Suya preparation process in Nigeria lacks standard or hygienic quality control; therefore, there is increase in food safety risks (Odusole and Akinyanju, 2003; Inyang et al., 2005). Its microbiological and parasitic quality assessment is therefore necessary in order to determine its safety vis-à-vis the consumption level. Suya contamination to unsafe level at the point of consumption with air flora and other microorganisms from handlers, equipment, utensils like trays, spoons and knives is possible, although epidemiological evidence on outbreaks of suya-borne diseases is scarce. Pathogenic microorganisms have been implicated in suya and other roasted meats products (Abdullahi et al., 2004; 2006; Edema et al., 2008; Ogbonna et al., 2012).

National Agency for Food and Drug Administration and Control (NAFDAC) and Standard Organization of Nigeria (SON) are the main regulatory bodies of food products in Nigeria; however, observation shows that most suya processsors do not follow the recommended tenets of these organizations and fall short of the dictates of HACCPs and ISO recommendations.

Most studies on suya have not considered the microbial quality of the meat during its processing, which requires the meat to be heated before consumption or investigated the parasitic protozoa of the meat. This study investigated the microbiological and parasitic quality and safety of suya sold in Makurdi, Nigeria.

MATERIALS AND METHODS

Suya sample

Two hundred and forty samples of ready-to-eat suya were purchased from suya vendors located in four different markets within Makurdi metropolis. Makurdi is the capital of Benue State, Nigeria, lying on latitude 7°30'N and longitude 8°35'E. Samples were collected using disproportionate random sampling technique, at the rate of 60 samples from each location. They were then analyzed. The vendors from each market location were visited six times at two-week intervals and ten samples were purchased from each location in every visit. The locations included low level, high level, Wadata and North Bank representing the major markets of Benue State capital. Samples were aseptically transported in sterile cellophane bags to the Microbiology and Parasitology Laboratories of the University of Agriculture, Makurdi for analysis. Each purchase was divided into three (A, B and C) portions. Group "A" samples were analyzed within 1 to 2 h after purchase. Group "B" samples were left for 6 h at ambient temperature before analyses. Group "C" stayed for 6 h at ambient temperature, re-roasted for 5 min and cooled further to room temperature (30 ± 1°C) before analysis.

Microscopic examination of parasites

Ten gram of ready-to-eat suya weighed and added to 90 mL of sterile saline-peptone-water was mixed by shaking vigorously with hands for 5 min in a sterile 250 ml screw-capped bottle. Thereafter, it was left untouched for 30 min and further diluted to up to 10^{-6} concentrations. A drop of the suspension was placed on a glass slide; it was covered with a cover slip and viewed under a microscope using xl0 and x40 objective lenses in order to identify possible cyst or egg of parasites. The parasites were identified based on the method of Ash et al. (2000).

Microbiological analyses

Microbiological analysis involved weighing 10 g of suya, cutting into pieces using sterile pair of scissors and adding to 90 ml of sterile saline-peptone-water (8.5 g L^{-1}NaCl and 1 g L^{-1} peptone) solution. The mixture was shaken vigorously with hand for 5 min in a sterile 250 ml screw-capped bottle. It was left untouched for 30 min and further diluted up to 10^{-6} concentrations. Media were sterilized by autoclaving, and Desoxycholate Citrate Agar (DCA) was boiled. Each medium was inoculated with a 0.1 ml quantity of appropriately diluted suya sample, surface plated and incubated at 37°C for 24 h. Emerging colonies were counted using digital colony counter (Labtech, New Delhi, India) and multiplied by the dilution factor. Counts were expressed as log CFU/g.

Isolation and enumeration of bacteria

Aerobic colony count

Nutrient Agar (NA) (Biotech Lab. Ipswich, UK) was inoculated with a 0.1ml quantity of appropriately diluted suya sample and incubated at 37°C for 24 h. Colonies obtained after incubation were counted using digital colony counter (Labtech, New Delhi, India) and counts were multiplied by the dilution factor. Counts were expressed as log CFU/g.

Coliforms

Isolation and enumeration of coliform were done on MacConkey agar. A 0.1 ml quantity of appropriately diluted suya sample (between 10^{-1} to 10^{-6} concentrations) was surface-plated and incubated at 37°C for 48 h. Thereafter, colonies showing fermented lactose were counted as previously described and expressed as $\log_{10}$ CFU/g.

E. coli

Isolation and enumeration of *E. coli* was carried out on Eosine Methylene Blue Agar (EMBA) (Himedia Laboratories Pot Ltd, India) plates, which were inoculated as described previously and incubated at 37°C for 48 h. Typical colonies with greenish and blackish metallic sheen were subjected to biochemical tests for *E. coli*. Positive indole test was used to identify *E. coli* from the colonies with the metallic sheen. Colonies were counted as described above and expressed as $\log_{10}$ CFU/g.

S. aureus

Isolation and enumeration of *S. aureus* was done on a sterile Baird Parker Medium (BPM) (Lab M. Ltd, Bury Lancashire BL9 6As, United Kingdom), incubated at 37°C for 48 h. Greyish-black or black colonies with or without a halo were presumptively identified as Staphylococci (Macfaddin, 1977). Furthermore, coagulase test was carried out to further characterize *S. aureus*.

Salmonella* and *Shigella sp

Desoxycholate Citrate Agar (DCA) (Park Scientific Limited, Moultan Park, Northampton) was used for the isolation and enumeration of *Salmonella* and *Shigella sp.* A 1.0 ml quantity of suya saline-peptone-water mixture was inoculated on 9 mL of pre-enrichment broths (tetrathionate and selenite cysteine) and incubated at 37°C as recommended by Macfaddin (1977). DCA plates were inoculated with 0.1 ml of the pre-enrichment broth with 24 h growth and incubated at 37°C overnight (Macfaddin, 1977) and *Salmonella spp.* were identified as typical colonies with black centres. Pinkish colonies were identified presumptively as *Shigella* on DCA and subjected to further biochemical testing ((TSI) to confirm the organisms.

Bacillus

Nutrient Agar (NA) (Biotech Lab. Ipswich, UK), sterilized by auto-claving and cooled to about 45 – 50°C, was added to 10% human blood, and dispensed into sterile plates. Solidified plates were thereafter inoculated with a 0.1 ml quantity of appropriately diluted suya sample (between 10^{-1} to 10^{-6} concentrations), surface plated and incubated at 37°C for 24 h. Colonies were subjected to morpho-logical, microscopic and biochemical examinations to identify and enumerate *Bacillus*, based on the method of Macfaddin (1977).

***Klebsiella, Enterococcus, Streptococcus* and *Pseudomonas* sp.**

The organisms were isolated and enumerated on Nutrient and MacConkey agar and subjected to biochemical testing according to the procedures of Cowan and Steel (1965) and Cheesbrough (2004).

Biochemical identification of the bacterial isolates

The biochemical tests for the identification of the isolates were the IMVIC (indole, methyl-red, Voges-proskauer, citrate utilization), triple sugar iron (TSI), urease, oxidase, coagulase and catalase tests. The procedures earlier described were used for biochemical identification (Cowan and Steel, 1965; Macfaddin, 1977; Cheesbrough, 2004). The indole production test involved the inoculation of sterile peptone water with the test organism followed by incubation at 37^{o}C for 48h, where Kovacs' reagent shows a red colour in the reagent layer. This indicates the presence of indole. Methyl red and Voges-Proskuer tests were also performed following the procedure of Cowan and Steel (1965), where red colour stands for positive methyl red and yellow colour for negative. The citrate utilization test involved heavy inoculation of tryptose-citrate medium from a 10-12h culture and incubating in 40^{o}C water bath. After 90 min, 1 drop of 0.05% aq. bromothymol blue was added to each tube. A positive test was shown by blue colouration, whereas a green colour showed a negative result. Catalase test was conducted by immersing the cultured isolate on a clean oil-free glass slide, followed by the addition of few drops of hydrogen peroxide solution. An immediate effervescence showed positive test, whereas no visible reaction was negative. A yellow butt with a pinkish slant region and a trace of black precipitate indicate H_2S production was positive for TSI test. Colours for positive oxidase and urease were dark purple on a paper within 10s and reddish colouration respectively.

Yeasts and moulds counts

Potato Dextrose Agar (PDA) (Lab M. Ltd, Bury Lancashire BL9 6As, United Kingdom) plates were used for yeasts and mould counts. PDA medium containing 1% lactic acid was sterilized by autoclaving and inoculated as described above. Incubation was at 28°C for 5 days. Colonies were counted and expressed as $\log_{10}$ CFU/g.

Identification of the fungal isolates

Fungal identification was based on their colonial and morphological appearances using the parameters of size, colouration, nature of surface and edge as described by Alexopoulus et al. (1996).

Moisture content (%)

Ten gram of suya was weighed and dried to constant weight in an oven at 80°C; it was later reweighed. The moisture content calculated as the difference in weight between the fresh sample and the oven-dried sample was expressed as percentage (%) of the total weight of the sample:

$$MC = \frac{LWS}{WFS} \times 100$$

Where, MC = Moisture content (%), LWS = loss in weight of sample and WFS = weight of fresh sample.

Statistical analysis

Multiple-Sample Comparison, using STATGRAPHICS Centurion XVI Version 16.1.05 (32-bit), analyzed data. When the F-test in the ANOVA was significantly ($P<0.05$) different between the means, Multiple Range Tests were conducted to find the means that were significantly different from others.

RESULTS

Table 1 presents the mean values of microorganisms, eggs and cysts of parasites and moisture contents of suya from the different markets. The moisture contents showed percenttages between 32.00 and 37.80%. High Level samples left at ambient temperature for 6 h had the highest moisture content whereas low level samples without any storage had the lowest moisture content. There was no significant difference ($p>0.05$) between the mean moisture contents obtained from the different markets and between treatments. Moisture content increased with storage and slightly decreased in suya re roasted for 5 min following storage. Aerobic colony count of 7.27 Log_{10}CFU/g suya was the highest in the present study from group B samples of high level market area, whereas 4.91Log_{10}CFU/g suya was the lowest and was obtained from Wadata. There was a significant difference ($p<0.05$) between the total viable counts obtained from some of the markets. Coliform count was highest at 4.32 Log_{10}CFU/g suya in samples from high level and left at

Table 1. Mean value of microorganisms, eggs and cysts of parasites (expressed as Log_{10}CFU/g suya) and moisture contents of suya from the different markets.

Market	Sample group	Moisture content (%)	Aerobic colony count	Mean coliform count	Mean yeast and mould count	Mean parasite count
Low Level	A	32.00	6.08	4.07	2.05*	1.44
	B	36.00	6.26	4.31	3.42	2.85
	C	33.40	5.34	3.34	3.04	1.23
High Level	A	34.30	5.24	4.30	3.16	1.52
	B	37.80	7.27*	4.32	4.35	1.88
	C	35.50	6.45	3.07	3.18	1.33
Wadata	A	32.60	4.91*	3.26	2.51	ND
	B	34.50	6.10	4.07	3.54	ND
	C	34.00	6.01	2.23*	3.18	ND
North Bank	A	34.80	5.14	3.29	2.21*	1.45
	B	36.20	6.41	4.30	3.30	1.85
	C	35.10	6.39	3.17	3.05	1.75

[a]ND = not detectible; * = mean values significantly different ($P<0.05$).

ambient temperature for 6 h. The lowest coliform count of 2.23 Log_{10}CFU/g suya occurred in samples from Wadata in samples from Group C. Mean yeast and mould count ranged from 2.05 to 4.35 Log_{10}CFU/g suya. Mean values of 2.05 and 2.21 Log_{10}CFU/g suya obtained from low level and North bank were significantly lower than other mean yeast and mould. Mean Parasite Count ranged from not detectable to 2.85 Log_{10}CFU/g suya while samples from Wadata had no detectable eggs or cysts of parasites, high level samples had the highest number of eggs and cysts obtained in samples left at ambient tem-perature for 6 h before analysis.

Mean bacterial counts (expressed as Log_{10}CFU/g suya) of suya from the different markets is presented in Table 2. Mean *E. coli* counts ranged from 1.31 to 3.95 and samples from Wadata re-heated after storage had significantly lower counts of *E. coli* than others did. *S. aureus* mean count ranged from ND to 4.45 and suya from Wadata and North bank had no detectable *S. aureus*. The mean *Salmonella* counts also ranged from ND (in high level) to 4.84 (in North Bank) in sample exposed to ambient temperature for 6 h. The highest mean *Klebsiella* count was 3.98 Log_{10}CFU/g suya (from Wadata), whereas *Klebsiella* sp. were not detectable in suya from Low Level. *Shigella* species was not detectable in Low level and High Level. North Bank had the highest *Shigella* count of 2.99 (Table 2). *Shigella* count was relatively low in the area of study. Mean counts of *Enterococcus* ranged from ND to 4.99 Log_{10}CFU/g suya. Exposed meat sample had the highest level of *Streptococcus* of 4.26 Log_{10}CFU/g suya got in North Bank area. Wadata had no detectable *Streptococcus*. *Bacillus* sp. was isolated from all the market samples. The mean counts ranged from 1.20 to 5.15 Log_{10}CFU/g suya. Mean *Pseudomonas* sp counts ranged from not detectable to 3.83 Log_{10}CFU/g suya.

Eggs and cysts of parasites isolated from suya from the different markets (Table 3) showed that *Ascaris lumbricoides* was present in all the samples from high level but not detectable in samples from Wadata. The percentage occurrence of *A. lumbricoides* in suya in the study area WAS 50%. *Taenia* sp. was present in suya from low level, high level and North Bank but not detectable in samples from Wadata. *Taenia* sp. was the highest parasite isolated in the study area with percentage occurrence of 66.7. Eggs and cysts of *Entamoeba histolytica* were not detectable in suya from Low Level and Wadata but were very much present in samples from North Bank. The percentage occurrence of eggs and cysts of *E. histolytica* in the study area of 41.7 was moderateLY relative to other parasites. Eggs and cysts of Hookworm were the least isolated. Eggs and cysts of Hookworm seen in samples from High Level were not detectable in samples from other markets. The percentage occurrence of Hookworm was 16.7, which was the smallest of all the parasites encountered in the study area. Similarly, *Giardia lamblia* was present in low amounts with a percentage appearance of 25. It was not detectable in High Level and Wadata samples.

Yeasts and moulds were not detectible (ND) in many of

Table 2. Mean bacterial counts (expressed as Log_{10}CFU/g suya) of suya from the different markets.

Market	Sample group	Mean bacterial counts (expressed as Log CFU/g suya)								
		ECO	STA	SAL	KLE	SHG	ETC	STC	BAS	PDS
Low level	A	3.65	3.36	3.18	ND	ND	ND	3.00	3.88	2.60
	B	3.83	4.43	4.23	ND	ND	ND	3.70	5.15	3.08
	C	2.81	3.02	3.07	ND	ND	ND	2.34	4.36	2.07
High level	A	3.89	3.79	ND	3.62	ND	2.88*	ND	3.42	2.25
	B	3.95	4.45	ND	3.36	ND	4.99	ND	4.48	3.83
	C	2.36	4.15	ND	2.45	ND	4.68	ND	4.45	1.58
Wadata	A	2.45	ND	4.43	1.83*	1.34	1.89	1.48	1.34	ND
	B	3.49	ND	4.72	3.98	1.46	1.92	2.29	1.73	ND
	C	1.32*	ND	4.52	3.75	1.11	1.53	1.66	1.36	ND
North bank	A	2.90	ND	3.70	1.30*	2.89	3.34	3.45	1.20	1.00*
	B	3.78	ND	4.84	3.65	2.99	3.89	4.26	1.85	3.02
	C	2.16	ND	4.48	3.00	2.52	3.40	4.23	1.43	2.35

[a][ECO = *E. coli,* STA = *Staphylococcus aureus,* SAL = *Salmonella sp,* KLB = *Klebsiella sp,* SHG = *Shigella* sp, ETC = *Enterococcus* sp, STC = *Streptococcus* sp, BAS = *Bacillus* sp, PDS= *Pseudomonas* sp, ND = not detectible, * = mean values significantly different (P<0.05)].

Table 3. Levels of eggs and cysts of parasites isolated from suya from the different markets.

Market	Sample Group	*Ascaris lumbricoides*	*Taenia* spp	*Entamoeba histolytica*	Hook worm	*Giardia lamblia*
Low level	A	ND	++	ND	ND	+
	B	+	+++	ND	ND	++
	C	ND	+	ND	ND	ND
High level	A	+	+	+	+	ND
	B	++	++	+	+	ND
	C	++	+	ND	ND	ND
Wadata	A	ND	ND	ND	ND	ND
	B	ND	ND	ND	ND	ND
	C	ND	ND	ND	ND	ND
North bank	A	+	++	+++	ND	ND
	B	+	++	++	ND	+
	C	ND	ND	+	ND	ND
% occurrence		50	66.7	41.7	16.7	25

[a]ND = not detectible; + = present; ++ = moderately present; +++ very much present.

the samples (Table 4). *Aspergillus* sp. had the highest percentage occurrence (75%). Samples that stayed a period of 6 h at ambient temperature before analyses showed the highest % occurrence of yeasts and moulds.

DISCUSSION

The treatment processes used in this study were three and categorized into groups: A, B and C. Group “A”

Table 4. Occurrence of yeasts and moulds from the markets.

Market	Sample group	*Aspergillus* spp	*Penicillum* spp	*Rhizopus* spp	Y*easts*
	A	ND	ND	ND	ND
Low level	B	+	+++	++	++
	C	ND	ND	ND	ND
	A	+	ND	ND	+
High level	B	+++	++	+	+
	C	++	ND	ND	ND
	A	+	ND	++	ND
Wadata	B	++	++	+++	ND
	C	ND	ND	ND	ND
	A	+	ND	+++	++
North bank	B	+	++	+++	+++
	C	+	ND	+	ND
% occurrence		75.0	33.3	58.3	41.7

[a]ND = not detectible; + = present; ++ = moderately present; +++ very much present.

samples were analyzed within 1 to 2 h after purchase. Group “B” samples were left untouched for 6 h at ambient temperature before analyses. Group “C” stayed a period of 6 h at ambient temperature, re-roasted for 5 min and cooled further to room temperature (30 ± 1°C) before analysis. The consumption of suya, particularly in the study area and generally, in sub-Saharan Africa, falls into any of the following three groups: eating freshly prepared suya, eating stale suya and eating a re-roasted suya that has stayed for sometimes after preparation.

The percentage moisture contents (32 to 37.80%) obtained in the present study did not remarkably vary from the 35.00 to 39.09% obtained by Ogbonna et al. (2012) but was lower than the 40.17 and 57.17% obtained by Edema et al. (2008). Conversely, the range was higher than 23.29 obtained for kundi, another kind of dried meat (Fakolade and Omojola, 2008). During the preparation process of the meat, when enough heat is applied, it could reduce the moisture contents of the products and consequently lead to the reduction or elimination of the contaminating organisms. This however has the tendency to reduce the food nutrient. In all the samples, moisture content tends to increase with storage, which means that safeguarding the meat by immediate consumption as soon as it is prepared is necessary. However, there was no significant difference ($p>0.05$) between the mean moisture contents obtained from the different markets and between treatments. This implies that the regulation of the moisture content has to be during preparation.

Aerobic colony count of 7.27 Log_{10}CFU/g suya obtained in the present study is higher than the acceptable limits of 10^3 - $<10^4$ CFU/g (Gilbert et al., 2000). In addition, this value was higher than the following: 0.07 to 2.22 x 10^5 CFU/g , 10^5 to 10^6 and log_{10} 4.98 to log_{10} 6.27 reported by Edema et al. (2008), Inyang et al. (2005) and Abdullahi et al. (2006) respectively. The aerobic colony counts of a food product with 10^6CFU/g and above means that the food is contaminated to unsafe level and should attract public health attention. There was a significant difference ($p<0.05$) between the aerobic colony counts obtained from some of the markets. This could indicate lack of standardized processing and dispensing procedures.

Presence of coliform in food or drink is used as a yardstick for measuring the hygiene standard. The highest mean coliform count of 4.32 Log_{10}CFU/g suya in the present study is within the 10^4 CFU/g. This is taken as the tolerable limit of coliform in food in developed countries (Cooke and Gibson, 1990). This probably explains why people could consume the suya without necessarily getting down with infection. Coliform count did not vary significantly owing to exposure to ambient temperature. Since *E. coli* in particular is a specific coliform used as index of faecal contamination and sanitary quality, its presence between 1.31 to 3.95 Log_{10}CFU/g suya (Table 2) is disturbing. Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale show that *E. coli* ≥ 10^4CFU/g is unsatisfactory (Gilbert et al., 2000). The range of *E. coli* was lower than log_{10}4.08 CFU/g previously obtained (Abdulahi et al., 2006); this probably signifies better quality in terms of preparation and sales.

The mean count of *S. aureus* of the present study ranged from not detectable to 4.45 Log_{10}CFU/g suya and

was lower than 5.65 previously reported by Ogbonna et al. (2012). The mean *Salmonella* counts ranging from not detectable (in High Level) to 4.84 differed slightly from 1.97 x 10^5 CFU/g reported previously (Edema et al., 2008). Simonsen et al. (1987) suggested that to eliminate *Salmonella* in meat and poultry, it is necessary to maintain strict hygiene regimes and implement the HACCP concept. Public Health Laboratory Service Guidelines for the bacteriological quality of ready-to-eat foods at the point of sales consider a food unacceptable if the levels of *Salmonella* and *S. aureus* are in the order > 10^5 CFU/g and > 10^3 CFU/g respectively (Gilbert et al., 2000). *Shigella* count was relatively low in the area of study. *Shigella* species not detected in Low level and High Level signifies differences in hygiene standard of the markets. The highest mean *Shigella* count of 2.99 Log_{10}CFU/g suya of the present study (Table 2) was lower than the overall mean of 3.34 (log CFU/g of suya) reported by Ogbonna et al. (2012). Mean counts of *Bacillus* sp. ranged from 1.20 to 5.15 Log_{10}CFU/g suya. This signifies that some of the meats were unsafe for consumption since <10^3 is accepted as safe, and ≥ 10^5 CFU/g is recognized as unacceptable or potentially hazardous (Gilbert et al., 2000).

Klebsiella mean count of 3.98 Log_{10}CFU/g suya (from Wadata), *Enterococcus* mean count of 4.99 Log_{10}CFU/g suya, *Streptococcus* mean count of 4.26 Log_{10}CFU/g suya (from North Bank) and *Pseudomonas* sp. mean count ranging from not detectable to 3.83 Log_{10}CFU/g suya means that the four bacteria reached unsafe level in the suya. Similarly, that these bacteria were not detectable in some samples could mean that the major issue was hygienic standards.

Mean yeast and mould count ranged from 2.05 to 4.35 Log_{10}CFU/g suya which fell within log_{10}2.49 CFU/g reported by Abdulahi et al. (2006). It also slightly supports the value of the order ,10^4 to 10^5 CFU/g recorded by Edema et al. (2008). Mean values of yeast and mould obtained from Low Level and North bank were signifycantly lower than other mean yeast and mould. This also indicates lack of standard protocol.

Parasites in human system are responsible for a range of health-related problems. Mean Parasite counts ranging from not detectable to 2.85 Log_{10}CFU/g suya are high enough to cause health problems since they can multiply in human systems. The samples from some markets had no detectable eggs or cysts of parasites while others had signified differences in hygiene levels of the markets or processors. This is true since government inspectors are not able to inspect every animal that goes to the slaughterhouse in most developing countries. Other sources of the parasites to the suya could be from the processing water, including river, stream lakes and processors can use even pond water. Heating of the meat reduces the parasites' level (Table 3) but does not kill all of them. This means that further heating could completely eradicate the parasites but could also have negative effect on the food value.

The percentage occurrence of *A. lumbricoides* (50%) in suya in the study area, *Taenia sp* of 66.7%, *E. histolytica* of 41.7%, Hookworm of 16.7% and *Giardia lamblia* of 25% are significant to call for public health attention. The disparity in number of the parasites isolated could be due to differences in the processors' hygiene standard, the processing technology and the sales environments.

The baseline problem in Nigeria like most developing countries of the world is that the general hygienic provisions for primary production, general hygienic provisions for food establishments, registration and approval of food establishments and application of HACCP system as recommended by International Standard Organization (ISO) and HACCP (Arvanitoyannis et al., 2009) are either lacking or are inadequately implemented. Similarly, slaughterhouse hygienic requirements, role of the official veterinarian, recording of *ante-mortem* and *post-mortem* inspection results, professional qualifications of inspecttors, official control of slaughterhouses and good manufacturing practices (GMPs) (Arvanitoyannis et al., 2009) are not strictly observed. These could account largely for the high prevalence of these pathogenic organisms in suya.

Large populations of the inhabitants of sub-Saharan Africans consume suya. The present study shows that suya contains indicator organisms in the level that could endanger the lives of consumers. Similarly, there was no significant difference in the mean values of the organisms during pre and post-heat treatments. In general, to reduce the number of these pathogenic microorganisms and parasitic contents of suya, it is necessary to apply either HACCP or ISO 22000 along with prerequisite programs (Arvanitoyannis, 2009).

Conflict of Interests

The authors have not declared any conflict of interests.

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academicJournals

Vol. 8(35), pp. 3243-3249, 27 August, 2014
DOI: 10.5897/AJMR2014.7014
Article Number: D5BB99847150
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Occurrence and antimicrobial susceptibility of *Escherichia coli* and *Salmonella* spp. isolated from "zoom-koom" beverage and ice in Ouagadougou, Burkina Faso

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Received 15 July, 2014; Accepted 8 August, 2014

Pathogenic bacteria contaminate beverages and make consumers to have diarrhoeal diseases. This study was carried out to assess the occurrence and antimicrobial susceptibility of *Escherichia coli* and *Salmonella* spp. isolated from "zoom-koom" beverages and ice from open markets of Ouagadougou. One hundred samples of both "zoom-koom" and ice were analyzed using standard microbiological methods. Identification of serotypes of *E. coli* was completed using specific antisera. Strains were subjected to antimicrobial susceptibility test using agar diffusion method. *E. coli* was isolated in 36% "zoom-koom" and 35% ice samples. Among the 71 *E. coli* strains of both samples, there was 16.8% enteropathogenic *E. coli* (EPEC), belonging to six serotypes: *E. coli* O26, *E. coli* O55, *E. coli* O86, *E. coli* O119, *E. coli* O126 and *E. coli* O128. *Salmonella* was also found in 1% "zoom-koom" and 2% ice samples. Antibiotic susceptibility revealed that *E. coli* isolates resist amoxicillin-clavulanate, ticarcillin, cephalothin, cefamandole, ceftriaxone, cefepime, aztreonam, gentamicin, chloramphenicol, tetracycline, nalidixic acid and ciprofloxacin at different time. Meanwhile, *Salmonella* strains were sensitive to all tested antibiotics except tetracycline. The presence of EPEC and *Salmonella* spp. in the samples indicates that hygiene practices are being improved during processing to reduce the risk of having infection when they are consumed.

Keys words: "Zoom-koom"; ice, *Escherichia coli*, enteropathogenic *E. coli* (EPEC), *Salmonella*, antibiotics, Burkina Faso.

INTRODUCTION

Traditional beverages linked to the ancestral culinary practices in Africa have recently become popular in street trading in many developing countries such as Burkina Faso. "Zoom-koom" is a traditional sweetened beverage produced

from millet (*Penissetum glaucum*) flour. "Zoom-koom" produced essentially by handling and an unstandardized operation is one of common street-vended beverage. The millet grains are washed and dried. Some quantities of black pepper are added to them, after which they are ground into flour. The millet flour is then mixed with water. The resulting mixture is passed through a wet mesh sieve or a clean muslin cloth; this may sometimes be done in unhygienic environmental conditions, making it susceptible to contamination from flies. After filtration, ginger, lemon juice or pineapple and sugar are then added to the filtrate to give it taste, according to the will of manufacturer. 'Zoom-koom' is chilled in refrigerator or by using edible ice; then it becomes ready for consumption. It is usually sold using hand-filling glass cups and sometimes with hand tied in some disposable polythene bags or packaged in 0.5 to 1.5 L recycled plastic bottles. Many vendors reuse water to rinse the glass cups after each use. Poor handling practices, cross contamination during preparation and sales and ambient storage temperature could contribute to the presence of microbial pathogens in traditional beverages. The use of low quality water for the preparation of traditional beverages, reusing water to wash glass cups at the sales point, selling beverages without adequate protection and without good hygienic practices lead to pathogenic contamination (Barro et al., 2002; Lewis et al., 2006; Elmahmood and Doughari, 2007; Titarmare et al., 2009; Rashed et al., 2013). "Zoom-koom" is not only largely consumed for its characteristic aroma and nutritious value, but also for its non-alcoholic nature. Indeed, Compaore et al. (2011), in a previous study in Burkina Faso, showed that millet flour is rich in calcium, carbohydrates and proteins. It is also used in cultural and religious ceremonies such as baptisms and funerals in West African countries, especially in Burkina Faso. This beverage is often served with ice liberally to the thirsty customers.

Water is the most important raw material in the ice production business. Ice is often packaged and sold in transparent polythene nylon bags. Since ice used to refrigerate soft drinks is directly added to the beverages, it also needs to be safe for consumption (Falçao et al., 2002). When ice is thawed the microorganisms remaining may be injured, but they tend to recover their viability so that when the ice melts into drinks, they may be able to survive there too (Lateef et al., 2006). Many studies showed the association between contaminated ice, consumption of traditional beverages and food borne diseases due to pathogenic microorganisms such as: *Vibrio cholerae*, *Shigella* spp., *Salmonella* spp. and *Escherichia coli* (Agbessi et al., 2001; Falçao et al., 2002; Lateef et al., 2006; Mahale et al., 2008; Sunday et al., 2011).

It is now generally accepted that the main risk factor for the increase in resistance in pathogenic bacteria is the increased use of antibiotics. Use of antimicrobial agents in any environment creates selective pressures that favour the survival of antibiotic resistant pathogens (Nipa et al., 2011). The prevalence of antimicrobial resistance among food and beverages pathogens has increased in recent decades and become a major threat to public health. Previous studies have shown that ice and beverages can be contaminated with pathogenic bacteria (Lateef et al., 2004; Lateef et al., 2006; Rashed et al., 2013). Due to the substantial increase in resistance to antibiotics, it is essential to monitor the antibiotic susceptibility of pathogens in various food sold on the streets, especially traditional beverages and ice.

This study was designed to evaluate the prevalence and antimicrobial resistance of *Escherichia coli* and *Salmonella* serotypes in sample of "zoom-koom" and edible ice sold in Ouagadougou, the capital city of Burkina Faso.

MATERIALS AND METHODS

Study area and sampling collection

The study was conducted in the 18 major open markets in the five municipalities of Ouagadougou, from October 2011 to December 2012. In each of these markets, there are several sales points. One hundred (100) "zoom-koom" samples and one hundred (100) edible ice samples of approximately 300 ml were collected regularly in sterile containers; they were kept at 4°C and analyzed within 2 h after sampling. Each "zoom-koom "and ice samples was collected at the same points and time (Table 1).

Sampling processing

Isolation and identification of *Salmonella* and *Escherichia coli*

The ISO 6579:2002(E) and ISO 4832:1991 (F) methods lightly modified were used respectively for isolation and identification of *Salmonella* spp. and *E. coli*. Twenty-five milliliters (25 ml) of "zoom-koom" or edible ice samples were homogenized with 225 ml of sterile buffered peptone water (Liofilchem, Italy), and incubated at 37°C for 24 h. For *Salmonella* isolation, 0.1 ml of pre-enriched broth culture was homogenised in 10 ml of rappaport vassiliadis soy broth (Liofilchem, Italy) and incubated for 24 h at 42°C.

For *E. coli* isolation, two loopfuls of pre-enriched broth was streaked onto Eosin Methylene Blue (EMB) agar (Liofilchem, Italy) and incubated at 44°C for 24 h. Three to five presumptive metallic-green colonies were selected, purified by streaking onto Mueller Hinton agar (Liofilchem, Italy) and then tested by IMViC tests (Indole test, Methyl Red test, Voges Proskauer test and Citrate) and API 20E (bioMérieux, France).

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Table 1. Samples of "Zoom-koom" and ice in Ouagadougou markets.

Municipalities	Markets	Number of samples	
		"*Zoom-koom*"	Edible ice
Baskuy	"Oscar yaar"	5	5
	"Grand marché"	5	5
	"Samandin"	5	5
	"Laarlé"	8	8
	"Sankara yaar"	7	7
	"Hamdalaye"	10	10
	"Baskuy yaar"	6	6
Bogodogo	"Naby yaar"	5	5
	"Wemtenga"	6	6
	"14 yaar"	5	5
Boulmiougou	"Cissin"	5	5
	"Nonsin laafi yaar"	5	5
	"Pissy"	5	5
NongreMassom	"Somgande"	4	4
	"Tanghin"	3	3
	"Wayalghin"	6	6
Sig-Noghin	"Kilwin"	5	5
	"Tampouy"	5	5
Total	18	100	100

For *Salmonella* spp. isolation, two loopfuls of enriched broth were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Liofilchem, Italy) and incubated at 37°C for 18-24 h. Three to five suspected colonies (Red colonies with or without black centre) were selected and tested biochemically by using urease, indole test (BIO-RAD, France), orthonitrophenyl-β-D- galatopyranoside (ONPG), citrate, mannitol, mobility, hydrogen sulphur (H_2S) and fermentative gas production. Finally, the selected isolates were suspended in physiological saline solution (NaCl, 9 g/L) for confirmation by API 20E system (bioMérieux, France) and interpretation was done according to API 20E catalogue.

EPEC Serotyping

Enteropathogenic *E. coli* was confirmed by slide agglutination test using nonavalent, trivalent and monovalent antisera according to the instructions of the manufacturer (BIO-RAD, France). Twelve different serotypes were tested: trivalent I (O111 + O55 + O26), trivalent II (086 + O119 + O127), trivalent III (O125 + O126 O128) and trivalent IV (O114 + O124 + O142). O- antigen is detected by slide agglutination antibodies in the specific sera agglutinate with the bacteria when the corresponding antigens are present. Only strong agglutination occurring within 1 min was considered to be positive reaction.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for all isolates using the disc diffusion method as described by Bauer et al. (1966). The pure inoculum of *E. coli* or *Salmonella* spp. strains were prepared by suspending colonies into the physiological solution from agar plates and every suspension was adjusted to 0.5 McFarland standard. Diameters of inhibition zones were determined according to the European committee on Antimicrobial Susceptibility instructions (EUCAST, 2012).

The following antibiotic discs (Liofilchem, Italy) were classified according to ten classes: Aminopenicillins (amoxicillin-clavulanate, 30 µg); Carboxypenicillins (ticarcillin, 75 µg); Carbapenemes (imipenem, 10 µg); Monobactam (aztreonam, 30 µg); Cephalosporins (cephalothin, 30 µg), cefalexin (30 µg), cefamandole (30 µg), ceftriaxone (30 µg), cefepime (30 µg)); Aminoglycosides (gentamicin, 10 µg); Phenicoles (chloramphenicol, 30 µg); Cyclines (tetracycline, 30 µg); Quinolones (nalidixic acid, 30 µg); and Fluoroquinolones (ciprofloxacin, 5 µg). After incubation, the inhibition zones were measured and tested isolates were categorized as sensitive or resistant according to the EUCAST criteria. The inhibition zones of *E. coli* were controlled with the reference sensitivity of *Escherichia coli* ATCC 25922.

RESULTS

Occurrence of *E. coli* and *Salmonella* spp.

The results revealed that out of 100 "zoom-koom" samples examined, *Salmonella* spp. was present in 1 (1%) sample, while *E. coli* was present in 36 (36%) samples. It also showed that *Salmonella* spp. was found in 2 (2%) of the 100 samples of edible ice while *E. coli* was present in 35 (35%) samples (Table 2).

Table 2. Prevalence of *E. coli* and *Salmonella* spp. in "zoom-koom" and edible ice.

Traditional drinks	Number of samples	Number (%) of positive samples	
		E. coli	*Salmonella* spp.
"Zoom-koom"	100	36 (36.0%)	01 (1.0%)
Edible ice	100	35 (35.0%)	02 (2.0%)
Total	200	71 (35.5%)	03 (1.5%)

Table 3. O-serogroups of Enteropathogenic *E. coli* (EPEC) isolates in "zoom-koom" and edible ice samples.

Products	Number of *E. coli* isolates	Serotypes of EPEC isolates Number (%) of serotypes					
		O26	O55	O86	O119	O126	O128
"Zoom-koom"	36	0(0%)	0(0%)	2(5.5%)	1(2.7%)	2(5.5%)	1(2.7%)
Edible ice	35	1(2.8%)	1(2.8%)	2(5.7%)	1(2.8%)	0(0%)	1(2.8%)
Total	71	1(1.4%)	1(1.4%)	4(5.6%)	2(2.8%)	2(2.8%)	2(2.8%)

Table 4. Resistances of *E. coli* and *Salmonella* spp. isolated.

Antimicrobials	*Escherichia coli*		*Salmonella* spp.	
	ZK (n=36)	EI (n=35)	ZK (n=1)	EI (n=2)
	Number (%) of Resistance strains			
Amoxicillin- clavulanate	4 (11.10%)	7 (20.00%)	-	-
Ticarcillin	23 (63.90%)	20 (57.10%)	-	-
Cephalothin	1 (2.80%)	1 (2.90%)	-	-
Cefalexin	-	-	-	-
Cefamandole	2 (5.60%)	3 (8.60%)	-	-
Ceftriaxone	2 (5.60%)	4 (11.40%)	-	-
Cefepime	1 (2.80%)	2 (5.70%)	-	-
Imipenem	-	-	-	-
Aztreonam	8 (22.20%)	10 (28.60%)	-	-
Gentamicin	1 (2.80%)	2 (5.70%)	-	-
Chloramphenicol	3 (8.30%)	3 (8.60%)	-	-
Tetracycline	23 (63.90%)	21 (60.00%)	1 (100%)	2 (100%)
Nalidixic-acid	5 (13.90%)	5 (14.30%)	-	-
Ciprofloxacin	1 (2.80%)	1 (2.90%)	-	-

ZK, "Zoom-Koom"; EI, Edible Ice; -, None.

EPEC serotyping

Of the 71 (35%) isolates of *E. coli* in "zoom-koom" beverage and ice, EPEC was identified in 12 (16.8%) samples. Six (6) different serotypes of EPEC were identified: *E. coli* O86 (5.6%), followed by *E. coli* O119 (2.8%); *E. coli* O126 (2.8%) and *E. coli* O128 (2.8%). The less prevalent serotypes were *E. coli* O26 (1.4 %) and *E. coli* O55 (1.4 %). *E. coli* O26 (2.8 %) and *E. coli* O55 (2.8%) serotypes were only detected in edible ice while *E. coli* O126 (5.5 %) was detected in "zoom-koom" (Table 3).

Antibiotics susceptibility

The antibiotic resistance of the bacteria is shown in Table 4. It is noticeable that all *Salmonella* strains were sensitive to all tested antibiotics except two strains which were resistant to tetracycline. Antimicrobial susceptibility test revealed that all *E. coli* strains isolated showed no resistance to cefalexin and imipenem (100%). *E. coli* isolated from "zoom-koom" and ice was resistant to tetracycline (63.90 and 60.00% respectively) and ticarcillin (63.90 and 57.10%); it was moderately resistant to aztreonam (22.20

Table 5. Resistances of enteropathogenic *E. coli* serotyped isolated.

Antimicrobials	Serotypes of EPEC isolates											
	***E. coli* O26**		***E. coli* 055**		***E. coli* 086**		***E. coli* 0119**		***E. coli* 0126**		***E. coli* 0128**	
	ZK (n=0)	**EI (n=1)**	**ZK (n=0)**	**EI (n=1)**	**ZK (n=2)**	**EI (n=2)**	**ZK (n=1)**	**EI (n=1)**	**ZK (n=2)**	**EI (n=0)**	**ZK (n=1)**	**EI (n=1)**
	Number (%) of Resistance strains											
Amoxicillin-clavulanate	None	-	None	-	R(50%)	R(50%)	-	-	-	None	-	-
Ticarcillin	None	-	None	R(100%)	R(50%)	R(50%)	R(100%)	R(100%)	R(50%)	None	R(100%)	R(100%)
Cephalothin	None	-	None	-	-	-	-	-	-	None	-	-
Cefalexin	None	-	None	-	-	-	-	-	-	None	-	-
Cefamandole	None	-	None	-	-	-	-	-	-	None	-	-
Ceftriaxone	None	-	None	-	-	-	-	-	-	None	-	-
Cefepime	None	-	None	-	-	-	-	-	-	None	-	-
Imipenem	None	-	None		-	-	-	-	-	None	-	-
Aztreonam	None	-	None	R(100%)	R(50%)	R(50%)	R(100%)	R(100%)	-	None	R(100%)	R(100%)
Gentamicin	None	-	None	-	R(50%)	R(50%)	-	-	-	None	R(100%)	R(100%)
Chloramphenicol	None	-	None	-	-	-	-	-	-	None	-	-
Tetracycline	None	R(100%)	None	R(100%)	R(50%)	R(50%)	R(100%)	R(100%)	R(50%)	None	R(100%)	R(100%)
Nalidixic-acid	None	-	None	-	R(50%)	-	-	-	-	None	R(100%)	-
Ciprofloxacin	None	-	None	-	-	-	-	-	-	None	-	-

ZK, "Zoom-Koom"; EI, Edible Ice; -, None.

and 28.60%), nalidixic acid (13.90 and 14.30%), and amoxicillin-clavulanate (11.10 and 20.00%) and less resistant to chloramphenicol (8.30 and 8.60%), ceftriaxone (5.60 and 11.40%), cefamandole (5.60 and 8.60%), cephalothin (2.80 and 2.90%), gentamicin (2.80 and 5.70%), ciprofloxacin (2.80 and 2.90%), and cefepime (2.80 and 5.70%). Most EPEC strains are resistant to tetracycline, aztreonam and ticarcillin (Table 5).

DISCUSSION

The presence of *Salmonella* spp. in "zoom-koom" and edible ice samples indicates that consumers are exposed to infections. Our findings are relatively low than those reported in other studies on ice and fruits juice beverage (mango, orange etc) in Ivory Coast, Mexico and India (Agbessi et al., 2001; Falçao et al., 2002; Lewis et al., 2006). These data are not surprising because "zoom-koom" and ice are processed mainly by handling operations and without pasteurization treatment. Street-vended foods and beverages dominated by hand intervention without adhering to good hygienic practices can lead to contamination (Agbessi et al., 2001; Barro et al., 2002; Lewis et al., 2006; Elmahmood and Doughari, 2007; Sunday et al., 2011).

In our study, contamination of "zoom-koom" and edible ice caused by *E. coli* could be attributed to poor quality water and unhygienic practices during the production process. According to Makut et al. (2013), it is very possible that the pathogenic contamination could occur during hawking and improper or careless handling and packaging of the products. Indeed, Taylor et al. (2000) proved that the transfer of microorganisms to hands was due to poor personal hygiene after visiting the toilet. Our results on the presence of *E. coli* are in agreement with some of the earlier reports on its presence in fruit juices, soft drinks and ice in many developing countries (Barro et al., 2002; Falçao et al., 2002; Lateef et al., 2006; Lewis et al., 2006; Mahale et al., 2008; Uma Reddy et al., 2009; Sunday et al., 2011; Poojara and Krishna, 2012).

Regarding traditional beverages sold in markets, packaging inside a bag or recycled bottle requires a transfer. This manual transfer is sometimes done in the open air which causes the influx of flies that are likely to contaminate the product. In addition, a previously study conducted in Burkina showed that the flies landing on food or the surface of equipment can also spread bacteria, because they have contact with dirty matters (Barro et al., 2006).

Enteropathogenic *E. coli* (EPEC) serotypes, identified in our samples ("zoom-koom" and ice), cause severe foodborne disease (Stanilova et al., 2011). Indeed, it was reported that EPEC is transmitted by the fecal–oral route and is the major cause of infantile diarrhoea in developing countries (Clarke et al., 2002). According to Norazah et al. (1998) in Malaysia, EPEC presence in food indicated fecal contamination consecutive to unhygienic practices. The presence of EPEC in traditional drinks and edible ice could pose serious threats to the health of many consumers, like the "zoom-koom" and ice, which are highly consumed in Ouagadougou City. The markets are highly congested with people as a result, a single source of EPEC contamination could have widespread repercussion on people's health.

The rate of resistance obtained with antimicrobials tested is lower than those reported by Lateef et al. (2004) in orange juice in Nigeria and Lateef et al. (2006) in edible ice. This resistance of *E. coli* to beta-lactamin (amoxicillin-clavulanate, ticarcillin, ceftriaxone) could be explained by the production of beta-lactamase which hydrolyzes these antibiotics or the reduction of the structure of porins (Schwarz and Chaslus-Dancla, 2001). Also, the resistance to aminoglycosids such as gentamicin could be due to enzymatic activation by N-acetyltransferase and O-adenylyltransferase (Schwarz and Chaslus-Dancla, 2001). According to Lateef et al. (2004), the relatively high level of resistance to antimicrobial agents is a reflection of the misuse or abuse of these agents in the environment. The indiscriminate use of antibiotics, which promotes antibiotic resistance results from patients' demand, prescribers and dispensing doctors (Chinedum, 2005). Antimicrobial resistant strains of *Salmonella* spp. and *E. coli* isolated in this study constitute a serious public health problem.

From this study, it appears that "zoom-koom" and edible ice undergo fecal contamination during the various manufacturing processes. The presence of Enteropathogenic *E. coli* and *Salmonella* spp. strains in soft drinks and edible ice remains a significant public health concern. These pathogenic organisms have also shown to be highly resistant to antimicrobials tested, indicating a possible cause of public health hazards. Indeed, the ingestion of "zoom-koom" and ice contaminated with EPEC strains may cause the colonization of intestinal tract by antimicrobial resistant strains. Immediate action should be taken to spread awareness amongst the vendors about soft drinks safety and good hygienic practices in order to prevent contaminations and avoid any future pathogen outbreaks.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors expressed their gratitude to the "International Foundation for Sciences" for the funding provided for the study.

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Vol. 8(35), pp. 3250-3254, 27 August, 2014
DOI: 10.5897/AJMR2014.6648
Article Number: 965CFC847152
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Exploration of *Serratia entomophila* AB2 for lepidopteran pest control and productivity of groundnut

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Received 21 January, 2014; Accepted 8 August, 2014

The bacterial isolate *Serratia entomophila* AB2 having multidimensional attributes like larvacidal activity and nutrient-solubilizing capability was used to test its effect on productivity of groundnut. Two different inorganic carriers (talcum powder and vermiculite) based formulations of the bacterial isolate were also tested along with the unformulated product and 100% recommended dose of NPK (60:60:50) for comparative study. *S. entomophila* AB2 (unformulated) efficiently minimized lepidopteran pest infestations when compared with control (larval stage of *Heliothis armigera,* 27%; *Spodoptera litura*, 30%; *Plutella xylostella,* 23%). The strain also increased seed germination by 10%, increment in shoot weight was by 100% and enhancement of seed weight was by 120% in comparison to negative control in a field trial. But, both of the inorganic carrier based formulations showed better results in respect to pest control and productivity compared to the unformulated bacterium. Among the two formulations used in this study, vermiculite based formulation was found effective in terms of pest control and productivity. Consequently, a vermiculite based formulation of *S. entomophila* AB2 could be effectively used at the rate of 3.6 qthec^{-1} for quality and yield enhancement of groundnut.

Key words: *Serratia entomophila* AB2, fermentation, pesticide, nutrient solubilizer, integrated crop management (ICM) productivity.

INTRODUCTION

Several, bacterial entomopathogens have been developed for their use as commercial pest controlling agent and are *Bacillus thuringiensis*, *B. cereus*, *Burkholderia cepacia*, *Serratia entomophila*, *Pseudomonas fluorescens* (Johnson et al., 2001; Roh et al., 2009; Jeong et al. 2010; Sheen et al., 2013). But application of bacterial entomopathogens as soil inoculants is of rare occurrence. Among bacterial biopesticides *S. entomophila* was commercialized as soil inoculants to control pasture pest *Costelytra zealandica* (Coleoptera) in New Zealand (Johnson et al., 2001).

The strain *S. entomophila* AB2, used in this study, was reported with two unique features. Instead of controlling coleopteron pasture pests, *S. entomophila* AB2 was reported to control lepidopteron pests (*Heliothisarmigera*, *Spodopteralitura*, *Plutellaxylostella*) of phyllosphere region (Chattopadhyay et al., 2011, Chattopadhyay and Sen, 2012)

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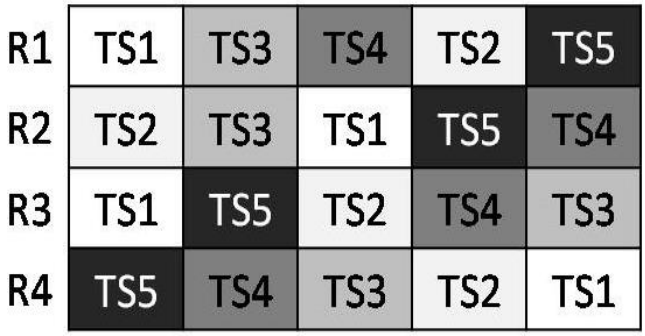

Figure 1. Randomized complete block design (RCBD): Experimental field showing different treatments (TS1, TS2, TS3, TS4 and TS5) with four replications (R1, R2, R3 and R4).

The strain was also characterized for P and Zn solubilization property (Chattopadhyay and Sen, 2012). Microbial organisms that solubilize nutrients is an important option for integrated crop management (ICM) that help to nourish the crop plants by increasing availability of soil nutrients (Gyaneshwar et al., 2002). Studies also proved systemic infestation of *S. entomophila* AB2 through plant parts to provide protection against pest and pathogen (Chattopadhyay and Sen, 2013). Therefore, the strain *S. entomophila* AB2 could be used in agriculture as potential pesticide (because of larvaecidal actiivity) and fertilizer (because of nutrient solubilization).

Limiting factors of using bacterial inoculants in field are related to storage, distribution of the product and requirement of large volume of water for application as suspension (Sabbour et al., 2012). Further, the use of biopesticide is limited for a couple of reasons but the most notable among them is the poor efficacy of the product under treatment conditions (Prior, 1989; Sabbour et al., 2012). To overcome these limitations, multitasking isolates with new formulations having extended shelf life and easy application are required to be developed.

In the present study, agricultural potentiality of *S. entomophila* AB2 was tested against groundnut, an important oil yielding cash crop highly susceptible to pest and pathogens (Adjou et al., 2012). Two different inorganic carriers (talcum powder and vermiculite) were tested for formulation. This communication makes the first attempt to understand the feasibility of a single indigenous strain, *S. entomophila* AB2, for pest control and productivity assessment in field condition for reducing the use of chemical pesticide and fertilizer.

MATERIALS AND METHODS

Experimental strain

The bacterial strain *S. entomophila* AB2 reported for its nutrient solubilizing, antifungal and larvicidal activity (Chattopadhyay et al., 2011; Chattopadhyay and Sen, 2012, 2013) was used. The 16S rRNA gene sequence was registered to Gene Bank (Accession no. GU370899). The isolate was maintained as 50% glycerol stock at -20°C in brain heart infusion agar (BHI-agar, Hi-media, India).

Fermentation

The working strain was grown in shake flask using 100 ml of broth (4 g sugar, 1 g yeast extract, 0.2 g urea and 0.2 g NPK; pH 7.1) as seed culture. Fermentation was carried at 28°C for 72 h in a glass fermenter (MCU-200, B.Braun Biotech International, India) at 240 rpm in same medium considering other parameters as per Visnovsky et al. (2008). Cells were harvested after they had entered the stationary growth phase.

Inorganic carrier used

For product formulation, talcum powder (magnesium silicate, $Mg_3Si_4O_{10}(OH)_2$) and vermiculite (Phyllosilicate, $(MgFe,Al)_3(Al,Si)_4O_{10}(OH)_2 \cdot 4H_2O$) were used as inorganic carriers. Sodium salt of carboxymethyl cellulose (CMC), generally referred as cellulose gum, was added as an adhesive agent.

Product formulation

After repeated sterilization, 80 g of carrier material was mixed with 18 ml of fermented broth (1.5 x 10^{10} cfu ml^{-1}), glycerol (1 ml, 50% v/v) and CMC solution (1 ml, 0.1 mg ml^{-1}) aseptically and uniformly to generate 100 g of product (Vidhyasekaran and Muthamilan, 1995). The formulation was shade dried aseptically to reduce the moisture content to ~18% and packed with in sterilized polythene bags under UV radiation and sealed. The formulation contained 3.5 x 10^8 $cfug^{-1}$ of experimental bacterial load when packed. The serially diluted sample was plated onto caprylate thallous agar (CTA) medium (O'Callaghan et al., 2002) supplemented with antibiotic Ampicillin (A) and Gentamicin (G) to measure the viable AB2 population from the packed formulation at intervals of 10 days.

Field trials

Soil conditioning experiments were conducted in Ravi season in experimental field (red laterite soil), keeping the field idle for 6 months prior to seed sowing for avoiding effects of any pesticide. Plots of 3.5 m x 4.0 m were laid out and brought to a fine tilt by ploughing. Soils of the plots were mixed well ensuring leveling and rows were made in 30 cm apart. Randomized complete block design (RCBD) model was followed for the experiments (Figure 1). Untreated (TS1) experimental plots were taken as control, whereas other plots supplied with 100% recommended dose of NPK (60:60:50) (TS2) were also maintained. Plots with soil conditioning was done with unformulated experimental strain (TS3), 90 ml 1.5 x 10^{10}cfu ml^{-1} cultures mixed with 5 Kg of powdered soil to broadcast over one plot area (4.0 m x 3.5 m). For talcum powder based (TS4) and vermiculite based (TS5) formulations, 500 g of formulated product (having 3.5 x 10^8 $cfug^{-1}$) was mixed with 4.5 kg of powdered soil to broadcast over one plot area (4.0 m x 3.5 m). All experimental plots were irrigated, as required to maintain the moisture level at 15%. In each case, treatment was carried out one hour before sunset (Ghidiu and Zehender, 1993).

Seed germination assessment

Seeds of ground nut (*Arachis hypogaea* var. Koushal, G201) were

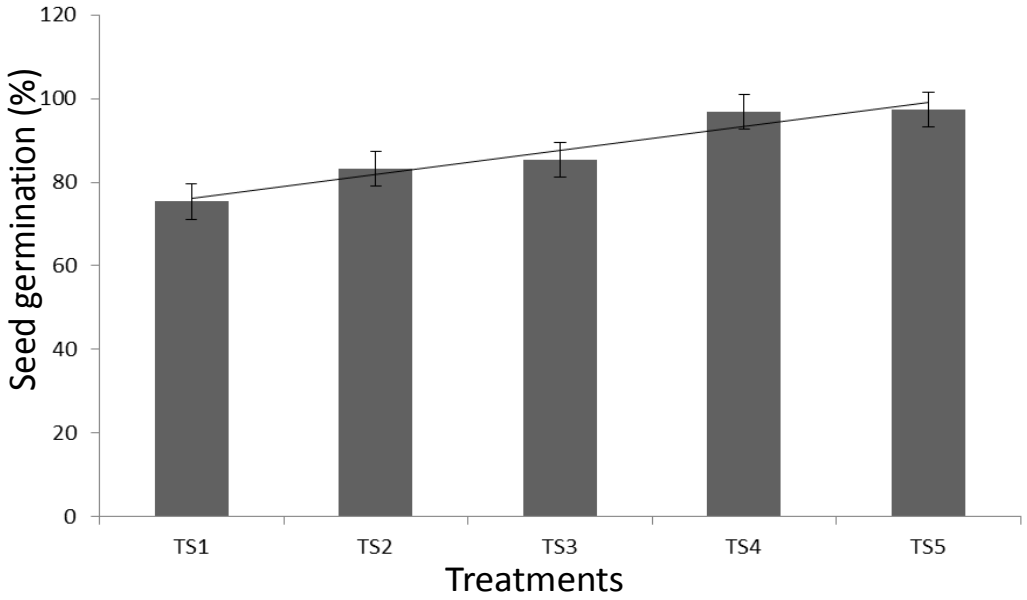

Figure 2. Effect of field treatment with, control (TS1), 100% NPK (TS2), unformulated strain (TS3), talcum powder based formulation (TS4) and vermiculite based formulation (TS5) on seed germination of ground nut.

surface sterilized and soaked in distilled water for 10 min and sowed in rows at a distance of 20 cm between the two as shown in Figure 1. After 10 days of sowing of seeds, seed germination percentage was recorded.

Pest control assessment

Experiments were carried out in open fields; therefore, the experimental field could have been infested by different pest naturally. Only larvae of lepidopteran pests, particularly *Heliothis armigera*, *Spodoptera litura* and *Plutella xylostella* were enumerated because the experimental strain is a known pathogen to these pest species (Chattopadhyay and Sen, 2012). Data was recorded since the 30th day of seed sowing till the period of experiment.

Productivity assessment

For productivity assessment, growth and yield parameters were measured. Different growth parameters were average measurement of branch number (BN), shoot length (SL), and shoot weight (SW) per plant. The plants were air dried for a period of 7 days for measuring dry weight. The yield parameters taken into consideration were average pod number per plant (PN), seed number per pod (SN), and seed yield per experimental plot (SY).

Statistical analysis

Standard deviation for each treatment was determined from four replications. The experimental data were statistically analyzed using ANOVA. Duncan's multiple range test (DMRT) was used to determine group mean value when ANOVA was found significant at $P < 0.05$. Pesticidal activity was evaluated, through pest scouting and mortality rate evaluation, on the basis of severity of infestations (Amer et al., 1999).

RESULTS AND DISCUSSION

Effect on seed germination

The rate of seed germination in different soil treatments was observed (Figure 2). It was found that the rate was much low in TS1 (75.4%) and in TS 2 (83.2%) While seeds of TS3 (85.4%) showed lower rate of germination than formulations (TS4 and TS5), causing almost 100% germination (96.8 and 97.4% respectively).

Effect of microbial consortium for seed germination is well studied (Pandey and Maheshwari, 2007; Babalola et al., 2007; Chen and Nelson, 2008; Naik and Sreenivasa, 2009). Formulations of *Pseudomonas* was used and showed significant increase of seed germination in *Vigna mungo* (Sarma et al., 2009a). Similar trend was achieved through application of the working isolate *S. entomophila* AB2 in seed germination of ground nut.

Effect on pest control

Highest pest attack was evident in plots treated with NPK (60:60:50) (TS2) which was found 119.56% more in comparison to control (TS1) (Figure 3). The results showed that *S. entomophila* AB2 (unformulated) efficiently minimized lepidopteron pest infestations when compared with control (*Heliothisarmigera,* 27%; *Spodopteralitura,* 30%; *Plutellaxylostella,* 23%). Among the experimental plots least pest attack was found in vermiculite based formulation of *S. entomophila* AB2 (TS5) (176.92% less pest attack thanTS2).

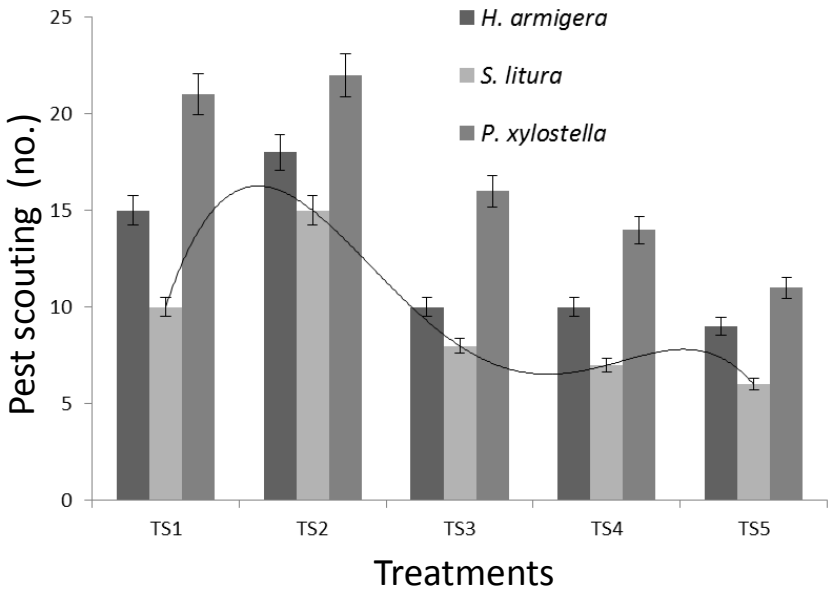

Figure 3. Effect of field treatments with control (TS1), 100% NPK (TS2), unformulated strain (TS3), talcum powder based formulation (TS4) and vermiculite based formulation (TS5) on providing protection against lepidopteran pests.

Table 1. Effect of *S. entomophila* AB2 and its formulations on productivity of ground nut.

Treatment	Branch number	Shoot length (cm)	Dry shoot weight (kg per experimental plot)	Pod number per plant	Seed number per pod	Seed yield (kg per experimental plot)
TS1 (control)	3.8±0.2	55.33±0.4	56.45±0.4	12.8±0.3	1.27±0.05	16.44±0.3
TS2(100% NPK)	6±0.3	61.33±0.5	70.2±0.7	20.5±0.3	1.9±0.05	40.08±0.4
TS3(unformulated strain)	4.5±0.25	63±0.5	70.23±0.7	18.9±0.3	1.5±0.05	35.66±0.4
TS4(talcum powder based formulation)	5.7±0.3	65±0.5	104.46±0.7	21.93±0.3	2.1±0.05	42.83±0.4
TS5 (vermiculite based formulation)	4.1±0.2	71.6±0.7	138.12±0.8	25.1±0.3	2.25±0.06	62.89±0.5

The value were presented as mean value of four replication ± standard error.

It was reported that broadcasting of talcum based formulation of *P. flurescens* strains (Pf1 and FP7) on paddy field significantly reduced sheath blight, thereby, increasing yield (Nandakumar et al., 2001). Similarly, the present study clearly demonstrated that, even the soil treated with *S. entomophila* AB2 alone (TS3) can provide an effective measure for controlling lepidopteron pest infection.

Effect on productivity

The observations recorded on plant growth in terms of BN, SL and SW, clearly indicated positive effect of the *S. entomophila* AB2 (Table 1). The strain (TS3) alone worked efficiently by 100% increment in shoot weight and 120% enhancement of seed weight in comparison to control in field trial. In growth experiments, both the formulations (TS4 and TS5) showed profound effect. Particularly, the vermiculite based formulation (TS5) showed maximum effect (129.47% increment in shoot length and 244.67% increment in shoot weight, in comparison to the control). Further, the SY was found maximum with vermiculite based formulation (TS5) and 156.91% increment in ground nut production was achieved in comparison to TS2 where soil was treated with NPK (60:60:50) (TS2).

The results show that the yield criteria viz., PN, SN and SY were influenced by different treatments TS5>TS4>TS2>TS3>TS1. It indicates that formulations

may be required for better availability of the strain in the rhizosphere for longer time duration. Thus, the effective role of *S. entomophila* AB2 on growth and yield parameters was evident. From earlier reports formulations of fluorescent *Pseudomonas* strain R62 and R81 were known to increase plant growth and productivity significantly in field condition (Sarma et al., 2009b).

Conclusion

The strain *S. entomophila* AB2, as a single biological agent for integrated nutrient management (INM) and integrated pest and disease management (IPDM) may find its application as a lucrative alternative to chemical fertilizer, pesticides and fungicides in ICM. But before that, its field efficacy should be checked. In the present study results of the field application indicate that the strain could be used as a soil inoculant. However formulations may be required for better availability of the strain in the rhizosphere for longer time duration which requires further investigation. On the basis of the result of this study, it can be recommended that vermiculite based formulation of *S. entomophila* AB2 could be used at the rate of 36 kg per 1000 sqm (3.6 qt hec^{-1}) for better quality and yield in groundnut. This could be a remunerative recommendation as it could effectively reduce the cost of chemical fertilizers and pesticides.

Conflict of Interests

The authors have not declared any conflict of interests.

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academicJournals

Vol. 8(35), pp. 3255-3264, 27 August, 2014
DOI: 10.5897/AJMR2014.6788
Article Number: 739310E47154
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and characterization of *Chromobacterium violaceum* from a disused tin-mining lake in Malaysia

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Received 21 March, 2014; Accepted 22 August, 2014

During a survey of microorganisms from a disused tin-mining lake in Kampar, Perak, Malaysia, a distinct bacterium producing a purple pigment was isolated. The isolate was characterized by morphological observation, followed by a series of conventional biochemical tests, physiological tests, as well as antibacterial tests, and identified as *Chromobactertium violaceum.* It was a facultatively anaerobic, motile, Gram-negative bacillus. The identity of this bacterial isolate was verified by a phylogenetic analysis of its 16S rRNA sequence. The ecological, medical, pharmacological and industrial importance of this bacterium with its production of the purple pigment, violacein, was briefly discussed.

Key words: Environmental microbiology, freshwater ecology, opportunistic infection, pigmented bacteria, 16S rRNA gene.

INTRODUCTION

Tin-mining activities in Malaysia had been very active in the late 19th century. The main tin-mining areas were in the Kinta Valley of Perak State, which included districts such as Ipoh, Batu Gajah, Gopeng and Kampar (Shamshuddin et al., 1986). The tin-mining activities, however, had ceased over a hundred years ago and there are now numerous disused tin-mining lakes in the Kinta Valley, especially in the vicinity of Kampar. In these lakes, minerals and organic materials are of great abundance and encourage a rich diversity of life. The microbial communities in such aquatic system play a vital role in global ecosystem and human health (Saleem et al., 2011).

In an attempt to isolate bacteria from a tin-mining lake in Kampar, we found a distinct isolate which produced a purple pigment. This paper described the isolation and

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characterization procedures of this bacterium. Its identity to be *Chromobacterium violaceum* Bergonzini (Bergonzini, 1880) was verified by a series of morphological, physiological and biochemical tests. The phylogenetic relationship of this isolate was inferred by comparing its 16S rRNA gene sequence with that of similar taxa. Previous findings about the ecological, medical and industrial importance of *Chromobacterium* species were summarized and briefly discussed. This paper represents the first report of the occurrence of *Chromobacterium* in the disused tin-mining lakes of Kampar region.

MATERIALS AND METHODS

Source of materials

Water samples were collected into a sterile 250 ml Schott bottle from a disused tin-mining lake in Old Town, Kampar, Perak, Malaysia. The temperature of the lake water was measured using a thermometer.

Isolation of bacteria using the enrichment method

Water sample was transferred into a sterile 15 ml Falcon tube and centrifuged at 6000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was re-suspended in 3 ml nutrient broth. The broth was then incubated overnight at 37°C (Malghani et al., 2009).

Serial dilution, spread plate and streak plate

After incubation, the sample was serially diluted to 10^{-6} with phosphate buffer saline (PBS). Diluted samples were then spread onto nutrient agar plates and incubated at 37°C for 18 h (Lammert, 2007). The plates were checked for microbial colonies. Interesting isolates were picked and streaked on nutrient agar in order to get isolated single colonies.

Isolation of bacteria using the membrane-filtration method

Water sample was first filtered through regular filter paper (Whatman) to remove any unwanted substances such as algae and plant debris in the water. Then, 100 ml of filtered water sample was transferred into a sterile filtration unit as shown in Figure 1:1. The cellulose acetate millipore membrane of 0.2 µm pore size was used as the filter (Lammert, 2007). A vacuum pump was connected to the filtration unit to ease the filtration process. After filtration, the membrane was transferred into a sterile 50 ml Falcon tube containing 10 ml of PBS, which was then mixed well and followed by serial dilution as well as plating on nutrient agar.

Morphological examination

Bacterial isolates were examined for colony morphology, followed by Gram stain (Cappuccino and Sherman, 2013) and endospore stain (Chess, 2009). The isolates were also observed under wet-mount microscopy for motility.

Optimal growth temperature experiments

To find out the optimal temperature range for growth, bacterial isolates were streaked onto nutrient agar plates and incubated at various temperatures for 18 h. All the isolates were tested for growth at 4, 22, 37, 46 and 60°C.

Conventional biochemical tests

Presence of extracellular enzymes

Bacterial isolates were tested for starch hydrolysis, casein hydrolysis and fat hydrolysis by inoculating to specific agar media. Starch Agar, Milk Agar and Egg Yolk Agar were used to detect hydrolytic activities of amylases, proteases and lipases, respectively. Isolates were streaked on each specific medium and incubated at 37°C for 18 h. The cultures were checked for hydrolytic result, which was indicated by a clear zone surrounding the bacterial colonies (Lammert, 2007).

Other biochemical and physiological tests

Bacterial isolates were tested for a number of biochemical and physiological properties using various specific agar media following standard protocols (Cappuccino and Sherman, 2013; Chess, 2009; Lammert, 2007). The following tests were performed: Oxidation-Fermentation (OF) Tests (for glucose and sucrose), Citrate Utilization Test, SIM Agar Test (for hydrogen sulfide production, indole production and motility test), Catalase Test, Oxidase Test, and Triple-sugar Iron (TSI) Agar Test. The isolates were also streaked on Blood Agar for observation of hemolysis.

API assay

The API assay for bacterial enzymes (Humble et al., 1977) was carried out by using the API ZYM test strip from BioMeriux SA. Enzymes assayed were alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The assay was carried out according to procedures described in the user manual of the API ZYM test kit. The API test strips were incubated for 4.5 h before colour changes were recorded and interpreted.

Antibacterial activity

Antibiotic susceptibility tests using disk-diffusion method

Isolates were subjected to antibiotic susceptibility tests according to methodology described by Bauer et al. (1966). The standard antibiotic disks used were chloramphenicol, tetracycline, penicillin, nitrofurantoin and sulfametaphazole and trimeroprim. Isolates were swabbed onto Müeller-Hinton agar plates followed by putting the antibiotic disks on the agar surface. Plates were incubated at 37°C for 18 h. After incubation, the plates were checked for antibiotic susceptibility by measuring the zones of inhibition.

Assay for antimicrobial agent using agar-well diffusion method

Isolates were also assessed for antibacterial activity against four standard indicators species using agar well-diffusion method (Liasi et al., 2009). The four standard indicator species were *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633) and *Staphylococcus*

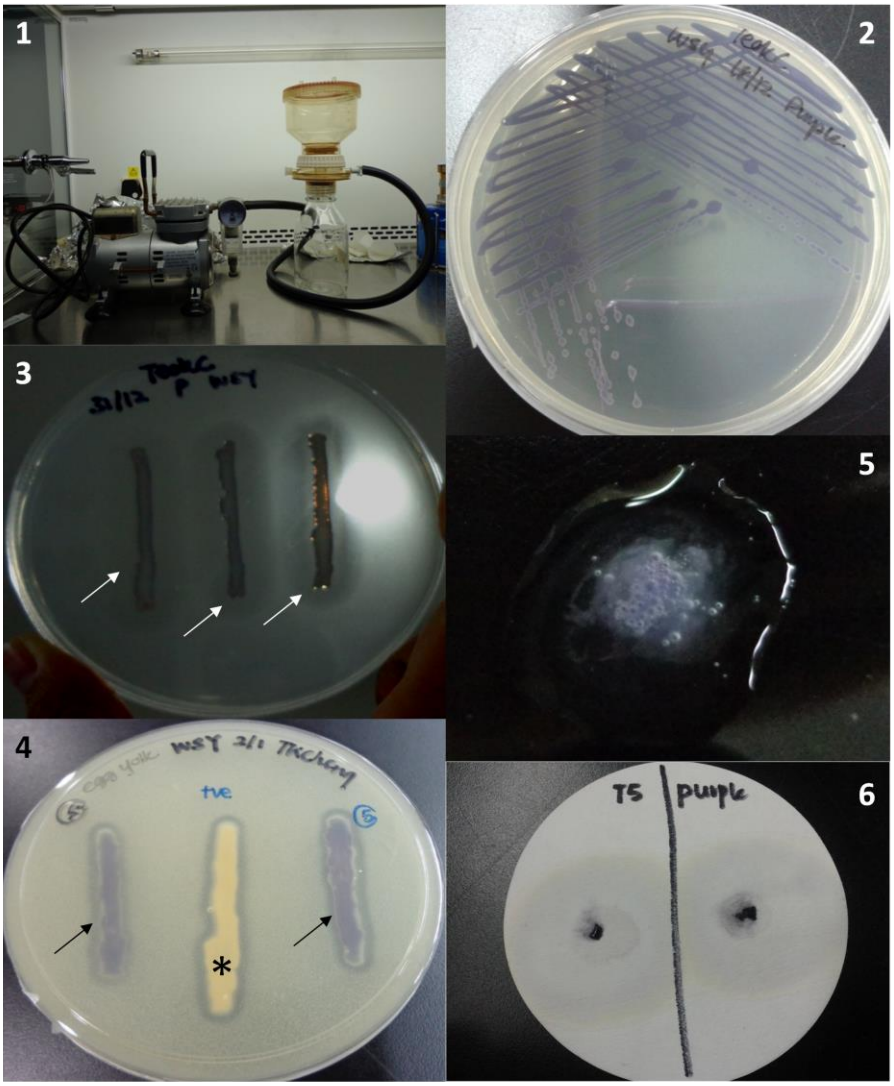

Figure 1. *Chromobacterium violaceum.* 1. Set-up of the membrane-filtration method for the isolation of bacteria from water samples. 2. Colonies on nutrient agar plate. 3. Casein Hydrolysis Test result (positive) in triplicates. Arrows point to clear zones due to hydrolysis. 4. Fat Hydrolysis Test result (positive) in duplicates. Arrows point to clear zones due to hydrolysis. Asterisk indicates position of positive-control isolate. 5. Catalase Test. Gas evolution indicates a positive result. 6– Oxidase Test. Test sample turns blue-black indicating a positive result.

aureus (ATCC 6538). Isolates were prepared in 0.85% saline. The bacterial inoculum's turbidity was referred to McFarland standard, for which the OD was within 0.1 and 0.2. Müeller-Hinton agar plates were punched with wells and the indicator species were swabbed onto the agar surface. 40 µL of the bacterial inoculum were transferred into the well, and the inoculated plates were incubated at 37°C for 18 h. After incubation, the plates were checked for the presence of zones of inhibition.

Phylogenetic analysis

Genomic DNA extraction

The genomic DNA of the present bacterial isolate (PB_Malaysia) was extracted using fast boil method (Holmes and Quigley, 1981). The bacterial samples were inoculated into LB broth for 24 h at 37°C with agitation of 200 rpm. After 24 h incubation, 1 mL of bacterial culture was centrifuged at 13,000 rpm for 5 min. The cell pellet was resuspended with 50 µL of sterile distilled water and incubated in water bath at 70°C for 20 min. After incubation, the sample was centrifuged at 13,000 rpm for another 5 min and the supernatant was transferred into a new sterile Eppendorf tube. The final concentration of the DNA was measured using Nanodrop 1000 (Thermo Scientific) and stored at -20°C for future use.

Polymerase chain reaction (PCR)

Partial 16S rRNA amplification was done by PCR with the genomic

DNA of isolate as template. The amplification reaction was made using the Taq DNA polymerase by 1st base and oligo 14-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTAGGACTT-3') as universal bacterial primers. PCR program was carried out in Thermal Cycler (Bio-rad) which comprised three steps: 1) denaturation at 94°C for 30 s; 2) annealing at 52.7°C for 30 s; and 3) extension at 72°C for 1 min. The amplicon was purified using a PCR purification kit (Philekorea Technology) and source out for sequencing analysis.

Phylogenetic analysis

The isolated 16S rRNA sequences were aligned and compared with available online database of other bacterial isolates using Basic Local Alignment Search Tool (BLAST) from National Center of Biotechnology Information (NCBI). The phylogenetic analysis was carried out based on the 16S rRNA sequences of the isolate being studied (PB_Malaysia). Neighbor-joining phylogenetic tree was constructed using MEGA 5.2 software and the bootstrap test was performed with 1000 replicates.

RESULTS

Growth and basic morphology

Water samples were collected from a disused tin-mining lake at Kampar, with water temperature ranging from 32 to 33°C. A number of different bacterial isolates were obtained from the collected water samples, among them, *Chromobacterium violaceum* was spotted easily due to its distinct purple colonies (Figure 1: 2). It was isolated from both the enrichment method and the filtration method. It grew well at 22 and 37°C, but not at 4, 46 and 60°C. The colonies on nutrient agar plates incubated at optimal temperature were purple in colour, circular, slight raised, with an entire margin and smooth surface. *C. violaceum* is motile, Gram-negative, non-sporing coccobacillus.

Biochemical and physiological properties

Our isolate of *C. violaceum* was able to hydrolyze casein (Figure 1: 3) and fat (Figure 1: 4), but not starch. It was catalase-positive (Figure 1: 5) and oxidase-positive (Figure 1: 6). It oxidized and fermented glucose (Figure 2: 7), but not sucrose. It was unable to utilize citrate as the sole carbon source. On SIM agar, it neither produced hydrogen sulfide nor indole, but it showed motility (Figure 2: 8).

On Blood Agar (Figure 2: 9), our isolate was distinctly β-hemolytic, showing clear zones surrounding the bacterial colonies (Figure 2: 10). On TSI medium, our isolate showed an alkaline slant and an acidic butt without H_2S or gas production (Figure 2: 11). Proteolysis and fermentation of glucose occurred, but lactose and sucrose were not utilized.

In API Test (Table 1 and Figure 2: 12), our isolate was positive in alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase. Table 2 summarizes the results of various biochemical tests of our *Chromobacterium* isolate in this study. The results of selected biochemical tests of our *Chromobacterium* isolate were compared with those of other isolates from other countries, based on the literature (Table 3).

Antibacterial activities

In the results of antibiotic disk-diffusion tests (Figures 3: 13 to 16), our isolate of *C. violaceum* showed susceptibility to chloramphenicol, tetracycline, nitrofurantoin and sulfametaphazole and trimeroprim. It was resistant to penicillin.

Our isolate of *C. violaceum* showed antibacterial ability to *Bacillus subtilis* subsp. *spizizenii* (Figure 3: 17) as well as *Staphylococcus aureus* (Figure 3: 18), but not *Salmonella typhimurium* and *Escherichia coli*

Molecular phylogenetic analysis

The 16S rRNA sequence analysis of our bacterial isolate showed a high identity (99%) with *C. violaceum*. The phylogenic tree (Figure 4) showed that our isolate clustered together with other *C. violaceum* isolates in a distinct clade supported by 99% bootstrap values. It was closer to, but distinct from *Vogesella indigofera*, another bacterium that produces a purple pigment.

DISCUSSION

The bacterium that was obtained from the disused tin-mining lake at Kampar, Malaysia, was identified as *C. violaceum*. Characterization of the isolate by various biochemical and physiological tests supported the identification of this bacterial species. Its identity was confirmed by our molecular phylogenetic analysis as well as the comparison of various biochemical test results with those reported from other countries (Antunes et al., 2006; Chang et al., 2007; Jitmuang, 2008; Kaufman et al., 1986; Kumar, 2012; Lee et al., 1999).

In phylogenetic studies, we compared our Malaysian isolate (PB_Malaysia) of *C. violaceum* with other pigment-producing bacterial taxa, and the identity of our isolate in *Chromobacterium* was unambiguous. The closest taxon to *Chromobacterium* is *V. indigofera* which produces a blue pigment (indigoidine). Although it is also commonly found in freshwater, it is not known to be pathogenic (Grimes et al., 1997). Other purple or blue pigment-producing bacterial taxa in our comparison were *Arthrobacter*, *Corynebacterium*, *Erwinia*, *Iodobacter* and *Janthinobacter* (Cardona-Cardona et al., 2010), but they were phylogenetically distant from *Chromobacterium*.

C. violaceum is a facultatively anaerobic, motile, Gram-negative bacillus. Currently there are a great number of

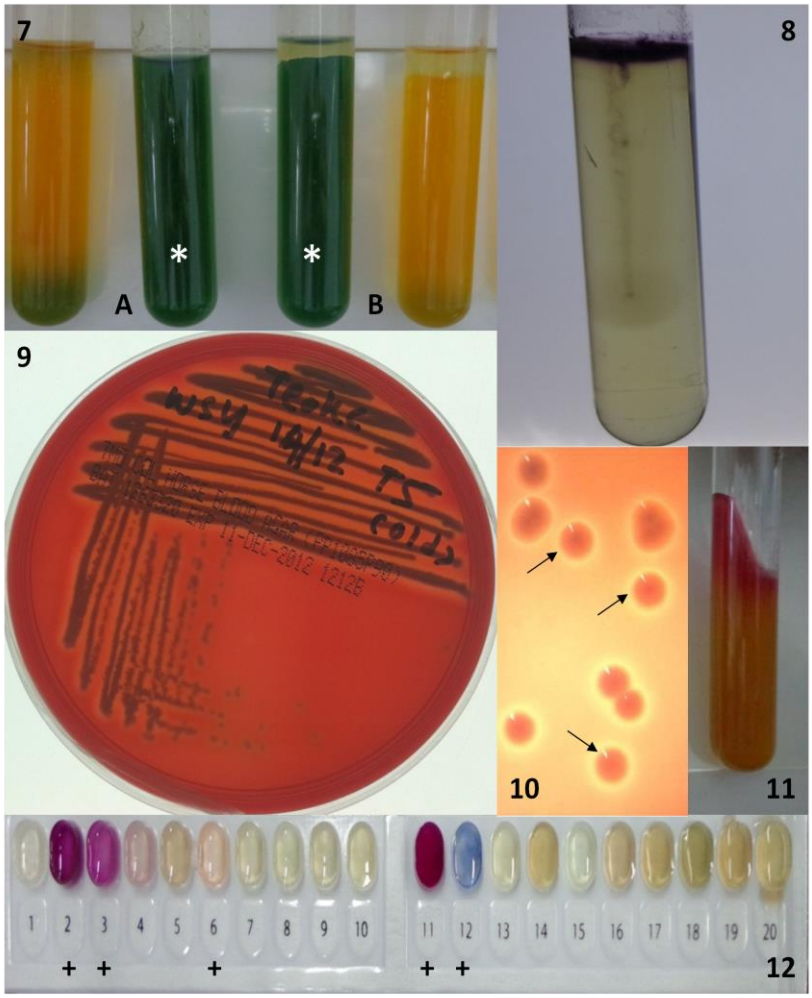

Figure 2. *Chromobacterium violaceum*. 7. Glucose Utilization Test result. A = aerobic (oxidative), positive; anaerobic (fermentative), positive. Asterisks indicate uninoculated control tubes. 8. SIM Agar Test result showing negative in hydrogen sulfide and indole production and positive in motility. 9-10. Colonies on blood agar showing b-hemolysis. Arrows point to clear zone due to b-hemolysis. 11. TSI Agar Test result showing alkaline slant (red), acidic butt (yellow) and no gas production. 12. Biochemical assay using the API test strip. Result show positive in alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase.

publications worldwide about this bacterium. It is a common inhabitant of soil and water confined to tropical and subtropical regions. Generally, it behaves as a saprophyte, but sporadically it becomes an aggressive opportunistic animal (including human) pathogen, causing serious infections with a high mortality in immune-deficient individuals. The production of violacean, the purple pigment, has attracted many researchers worldwide to study on this bacterium. With these many achievements in research on this bacterium throughout the decades, we therefore did not carry out any further research with our Malaysian isolate, but briefly presented in the following sections the various scientific findings, highlighting its medical, ecological and industrial importance.

Medical importance

In recent decades, *C. violaceum* has been noticed to cause severe systemic infection of humans, usually via an open wound (Jitmuang, 2008; Kumar, 2012). It is noteworthy for its difficult-to-treat entity characterized by a high frequency of sepsis and high mortality rate (Ang, 2004; Kaufman et al., 1986). The pathogenic potential of *C. violaceum* was first described by Wooley (1905) from a fatal infection in buffalo, while human infection was first reported in 1927 from Malaysia by Lessler (Sneath et al., 1953).

The most recent comprehensive review of human infections by *C. violaceum* worldwide was given by Yang and Li (2011). They studied 106 cases of *C. violaceum*

Table 1. Biochemical test results of *C. violaceum* using the API ZYM test strip.

Types of enzyme	Reaction
Control	–
Alkaline phosphatase	+
Esterase (C 4)	+
Esterase lipase (C 8)	–
Lipase (C 14)	–
Leucine arylamidase	+
Valine arylamidase	–
Cystine arylamidase	–
Trypsin	–
α-chymotrypsin	–
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α-galactosidase	–
β-galactosidase	–
β-glucuronidase	–
α-glucosidase	–
β-glucosidase	–
N-acetyl-β-glucosaminidase	–
α-mannosidase	–
α-fucosidase	–

Table 2. Results of various biochemical tests of *C. violaceum*.

Biochemical tests	*Chromobacterium* Isolate
Starch Hydrolysis Test	–
Casein Hydrolysis Test	+
Fat Hydrolysis Test	+
Oxidase Test	+
Catalase Test	+
Citrate Utilization Test	–
SIM Agar Test	
- hydrogen sulfide production	–
- indole production	–
- motility	+
Triple Sugar Iron Agar Test	Alkaline slant, acidic butt.
Oxidation-fermentation test	
- Glucose – aerobic	Glucose oxidized
- Glucose – anaerobic	Glucose fermented
- Sucrose – aerobic	No Sucrose oxidation
- Sucrose – anaerobic	No Sucrose fermentation

human infections from the literature, and provided a demographic data of the patients. Forty two percent of the cases were reported in the region of Americas (including Argentina, Brazil, Colombia, Guvana,and USA), 41% in the East Western Pacific (including Australia, Cambodia, Hong Kong, Japan, Korea, Laos,

Table 3. Comparison of selected biochemical test results of *C. violaceum* from different localities.

Biochemical tests	Test results of Malaysian isolate (this paper)	Test results of foreign isolates (from literature)	Localities and year of publications in previous studies*
Indole from SIM test	Negative	Negative	Brazil (2006), Taiwan (2007), Argentina (1986),Korea (1999), India (2012)
Motility from SIM test	Positive	Positive	Brazil (2006), Taiwan (2007), Argentina (1986)
Catalase	Positive	Positive	Brazil (2006), Thailand (2008), Korea (1999), India (2012)
Oxidase	Positive	Positive	Brazil (2006), Thailand (2008), Taiwan (2007), India (2012), Korea (1999)
TSI slant	Alkaline	Alkaline	Taiwan (2007), Argentina (1986), India (2012)
TSI butt	Acidic	Acidic	Taiwan (2007), Argentina (1986), India (2012)
Gas production from TSI test	Negative	Negative	Taiwan (2007), Argentina (1986), India (2012)
Hydrogen sulfide production from TSI test	Negative	Negative	Taiwan (2006), Argentina (1986), India (2012)

*Information regarding localities and year of publications are based on the following references: Argentina (Kaufman et al., 1986), Brazil (Antunes et al., 2006), India (Kumar, 2012), Korea (Lee et al., 1999), Taiwan (Chang et al., 2007), and Thailand (Jitmuang, 2008).

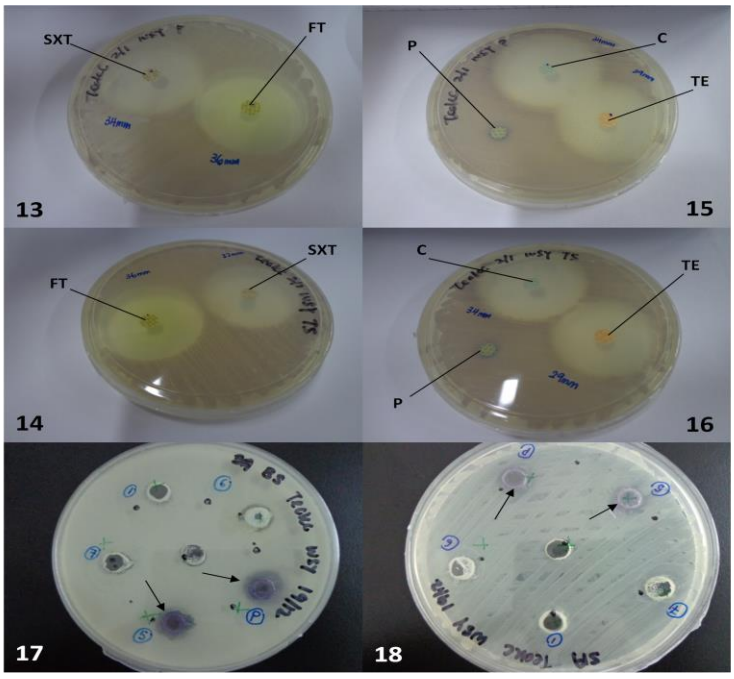

Figure 3. *Chromobacterium violaceum*. 13,14-Antibiotic susceptibility test by disk-diffusion method showing susceptibility to nitrofurantoin (FT) and sulfametaphazole and trimeroprim (SXT). 15, 16- Antibiotic susceptibility test by disk-diffusion method showing susceptibility to chloramphenicol (C) and tetracycline (TE), and resistance to penicillin (P). 17- Antibacterial activity test with *Bacillus subtilis* subsp. spizizenii (ATCC 6633) as the indicator species. Arrows point to inhibition zones against the indicator species. 18- Antibacterial activity test with *Staphylococcus aureus* (ATCC 6538) as the indicator species. Arrows point to inhibition zones against the indicator species.

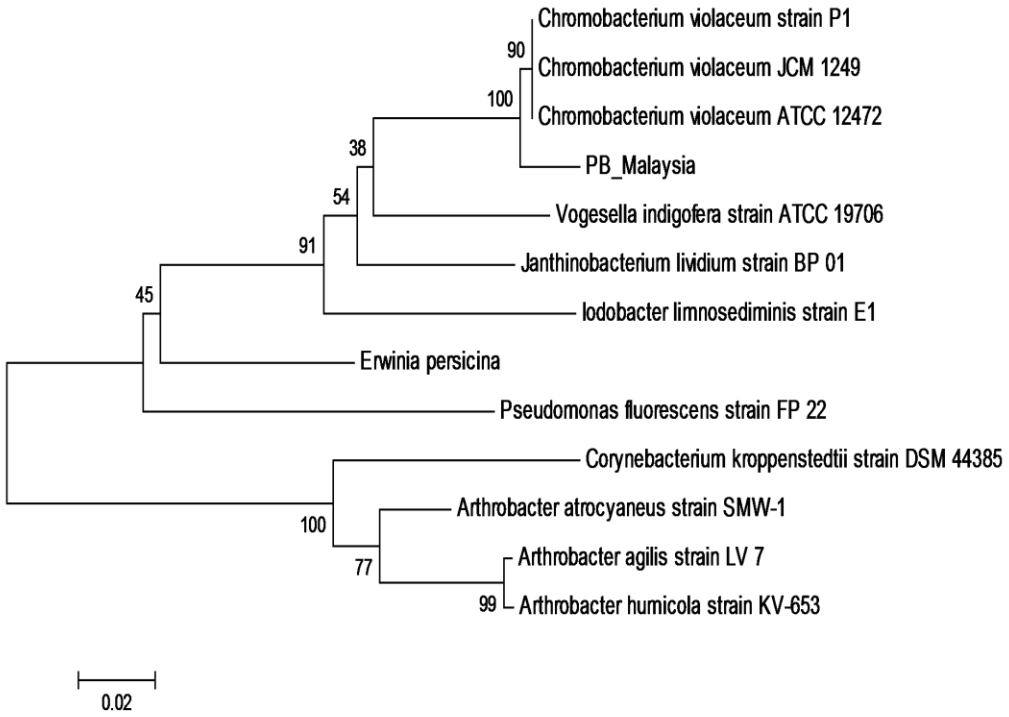

Figure 4. Neighbor-joining phylogenetic tree of *Chromobacterium violaceum* and similar bacterial taxa, based on the16S rRNA gene sequences. Bootstrap percentage values are given at the nodes of the tree. Bar represents 0.02 substitutions per nucleotide position. Result shows that the present Malaysian isolate of purple bacterium (PB_Malaysia) clearly clustered within the clade of *C. violaceum* with a bootstrap value of 100%.

Mainland China, Malaysia, Papua New Guinea, Singapore, Taiwan,and Vietnam). There were 16 reported cases in the South East Asia (including India, Sri Lanka, and Thailand), and 3 cases in Africa (including Nigeria and South Africa). Two other cases were recently added to the history: one from Italy (Arosio et al., 2011), and the other from Vietnam (Campbell et al., 2013). *C. violaceum* was known to be sensitive to temperature and thus it has a predilection to the tropical and subtropical areas (Saleem et al., 2011). Yang and Li (2011) commented that the effects of global warming may affect geographical distribution of this bacterium, and cases of infections may significantly increase in future.

Since the first report of *C. violaceum* occurrence in Malaysia in 1927 (Sneath et al., 1953), only during the past 20 years were additional cases of human infections by this bacterium recorded for this country (Saigin et al., 1994). Several local cases of *Chromobacaterium* infections were recorded in recent years, patients of which were children and adults, including a 4-year-old boy and an 11-year-old girl (Ang, 2004), 12-year-old boy (Sureisen et al., 2008), and a 45-year old man (Cheong, 2010). In Malaysia, fatal infections with *C. violaceum* were also observed in animals. Groves et al. (1969) reported infections in 9 gibbons and one Malayan sun bear in National Zoo, Kuala Lumpur. The disease symptoms in animals were similar to those in humans. Typical septicemic process with hepatic abscesses in all animals and pulmonary abscesses in most cases were reported. Our present paper represents the first record of occurrence of this bacterium in disused tin-mining lakes, but we did not encounter any infection in our study.

Biological activities of violacean and its pharmacological importance

The most notable characteristic of *C. violaceum* is the production of violacein. This purple pigment was first isolated in 1944 (Strong, 1944), and chemically characterized a few years later (Ballantine et al., 1958). This pigment is regarded as a natural antibiotic and hitherto there are a tremendous amount of literatures about this pigment. Work has been done to extract, purify, and produce the pigment in a larger scale (Rettori and Durán, 1998). Violacein is not diffusible, but soluble in ethanol and insoluble in water and chloroform (Moore et al., 2001). It has been used as an antimicrobial agent (Lichstein and Van de Sand, 1945; Durán et al., 1983; Durán and Menck, 2001), an insecticide and an anti-cancer agent (de Carvalho et al., 2006; Durán et al., 2007). It has been shown to have antifungal effect which

can protect amphibians from fungal infection (Becker et al., 2009). There are also research evidences of violacean that showed its successful inhibitory actions against certain pathogenic protozoa: against *Plasmodium* that causes malaria (Lopes et al., 2009), against *Trypanosoma cruzi* and *Leishmania amazonensis* (Leon et al., 2001). Violacean also has inhibitory effects against the nano-flagellates and some bacteriovorous protozoa (Matz et al., 2004). The versatility of violacean in terms of its bactericidal, tumoricidal, trypanocidal and antileishmanial activities was discussed by Leon et al. (2001). A potential application of its antiviral activity is discussed by Andrighetti-Fröhner et al. (2003).

Genetic analysis and violacein biosynthesis

C. violaceum has been reviewed as possessing many pharmacological and industrial perspectives (Durán and Menck, 2001). Many researchers were interested in cracking the genome of this bacterium (Brito et al., 2004). The complete genome sequence of this bacterium has revealed remarkable and exploitable biotechnological potentials (Brazilian National Genome Project Consortium, 2003). Molecular studies of the violacein-producing gene and the biosynthetic pathway of the pigment had been accomplished during the past decade (Antônio and Creczynski-Pasa, 2004; August et al., 2000)

Quorum sensing is a system of stimulus and response correlated to population density of organisms (Miller and Bassler, 2001). Bacteria use quorum sensing to coordinate certain behavior such as biofilm formation, virulence and antibiotic resistance. Production of violacean by *C. violaceum* has been used in studies of bacterial quorum sensing inhibition (Choo et al., 2006; McClean et al., 1997). Researchers are interested in the mechanism of molecular signaling in bacterial population. Recent studies by Anthony et al. (2013) explored the difference in quorum sensing regulation and violacein synthesis between the wild and mutant *C. violaceum*. They also have evaluated the reason behind the inhibition of violacein synthesis in *C. violaceum* mutant.

Biochemical, ecological and industrial importance

In the 1960's, *C. violaceum* was already known to produce polysaccharides that contribute to more stable soil structure in terms of enhancing humus production and affecting decomposition rate of soil (Martin and Richard, 1963; Corpe, 1964). This bacterium also exhibits certain chitinolytic activity which is involved in the regulation of quorum sensing (Chernin et al., 1998). A comprehensive account of the ecological versatility of *C. violaceum* was given by Hungria et al. (2004).

Cyanide-producing microorganisms are able to assimilate or detoxify cyanide by a variety of pathways (Knowles, 1976). Besides a few soil borne pseudomonads, *C. violaceum* is the only bacterium known to produce cyanide (Rodgers and Knowles, 1978). Moreover, *C. violaceum* also forms rhodanese as a possible detoxifying agent (Knowles, 1976). It is thus a potential agent for environmental bioremediation (Faramarzi et al., 2004).

Researchers in this century are keen to exploit intriguing microbial polymers. In a recent study by Bhubalan et al. (2010), a Malaysian isolate of *C. violaceum* was found to produce polyesters which can be used to make polyhdroxyalkanoates (PHA's). PHA's are an alternative to plastics made by petrochemicals. Cloning of gene in *C. violaceum* for the production of PHA has been accomplished by Bhubalan and his co-workers (Bhubalan et al., 2010).

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors wish to thank Universiti Tunku Abdul Rahman (UTAR) for providing research facilities and financial support in completing this study.

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Vol. 8(35), pp. 3265-3270, 27 August, 2014
DOI: 10.5897/AJMR2014.6891
Article Number: B00C3F347156
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Influence of bacterial species on adhesion to stainless steel

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Received 7 May, 2014; Accepted 22 August, 2014

To assess the adherence of bacteria in stainless steel used in food processing, we developed an experimental model of a milk circulation line equipped with coupons T, 90° (elbow) and cylindrical-shaped constructed AISI 304 stainless steel. We evaluated the adherence of *Enterococcus faecium*, *Pseudomonas aeruginosa* ATCC 15442 and *Bacillus cereus* NCTC 11145 in vegetative and spore forms, before and after milk circulation in the model. The micro-organisms were activated (35°C/12 h) in MRS to *E. faecium* and *B. cereus* and nutrient broth for *P. aeruginosa* and were used to inoculate milk so as to obtain a count of 1.0 x 10^6 CFU/ml and placed within the test coupons in order to fill them and subsequently incubation was performed at 18°C/12 h. It was concluded that percentages of adherent bacteria before milk circulation were significantly different ($p<0.05$) that is 13.6% for the *B. cereus* spores that adhered, 6.0% for the *P. aeruginosa*, 1.28% for vegetative and spore forms of *B. cereus* and 0.31% for *E. faecium*. There was no significant difference ($p\geq0.05$) between the micro-organisms that remained attached after the milk circulation. There was significant difference in removing bacteria, among coupons proof. No coupon T removal was higher than in cylindrical, probably due to the higher turbulent flow, whereas in the later, there is greater tendency of bacteria still attached.

Key words: Milk, adhesion, bacterium, biofilm.

INTRODUCTION

Industries have rendered increasingly larger amounts of food to meet the needs of the market. With the increase in processing capacity, several problems have emerged. One is the post-processing loss or reduced shelf life of food due to the contamination of food with micro-organisms attached to the production line. Since these microorganisms have adhered and biofilm formed on the surface of food processing, their removal or destruction will be much more difficult. Hence, the need for a good cleanup process which can remove microorganisms with

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efficiency. Microbial adhesion and subsequent biofilm formation occur in many fields of industrial and medical applications (Flemming, 2002; Von Eiff et al., 2005). About 99% of all bacteria in natural environments reside in biofilms that consist of microorganisms adhering to a surfaces with the aid of extracellular polymeric substances produced by the micro-organisms themselves (Jung et al., 2013). Bacteria adhering in dairy plant are resistant to the cleaning process with 200 ppm of chlorine for 30 min and contaminate subsequent batches of cheese (Somers et al., 2001). The production of exopolysaccharides increases when a bacterium adherent to a surface and is influenced by time and type of carbohydrate present in the food (Jung et al., 2013). It should be considered that milk is a complex substrate for microorganisms, the formation of biofilms is rapid since for instance, Gram negative bacteria easily multiply in milk residues after improper cleaning of milk equipment. Various factors affect the bacterial adhesion process and the formation of biofilms, including genotypic, thermodynamic and environmental factors (Renier et al., 2010). The genetic makeup of the organism, including the presence of flagella and fimbria, determines its ability to produce exopolysaccharides that assist in adhesion (Folson, 2006). Hydrophobicity and electric charge play an important role in the process of bacterial adhesion (Boks et al., 2008). Time, temperature, pH, the presence of alcoholic substances and the flow of cleaning solutions on the surfaces also actively participate in the process. The established biofilm matrices enable incorporation of pathogens like *Listeria monocytogenes*, which can cause a continuous contamination of food processing plants or another plant (Blackman and Frank, 1996). This microorganism is frequently found in raw milk and non-pasteurized raw milk products and as part of a biofilm community in milk meters and bulk milk tanks (Weiler et al., 2013). In a study performed to evaluate the adherence capacity of *Escherichia coli* O157: H7 stainless steel and high density polyethylene, researchers found that even at 4 or 12°C, this micro-organism can adhere and multiply on the surface of both surfaces studied (Dourou et al., 2011). In an experiment to induce the formation of biofilm FCM 40 of *Salmonella* in various materials used in the poultry industry in India, it was observed that after incubation at 28°C/48 h in tryptone broth inoculated with this bacteria, the biofilm had scores of 1.20×10^7, 4.96×10^6 and 2.23×10^5 CFU/cm^2 in high density polyethylene, concrete and stainless steel, respectively (Joseph et al., 2001). These authors also assert that this micro-organism cell in biofilms are more resistant to the action of chlorine to the planktonic cells and high density polyethylene was not possible to inactivate all the cells even with a 100 ppm treatment of Cl_2 for 25 min. Various micro-organisms have been studied with respect to their ability to form biofilm on surfaces in food processing, including *Staphylococcus aureus, Yersinia enterocolitica, Listeria monocytogenes* and *Campylobacter jejuni* (Weiler et al., 2013; Furukawa et al., 2010). The objective of this study was to assess the adherence of bacteria in stainless steel and its resistance to removal by the flow of food in a milk circulation model system.

MATERIALS AND METHODS

Microorganisms and culture media

The microorganisms used in this study were *Pseudomonas aeruginosa* ATCC 15442, *Bacillus cereus* NCTC11145 and *Enterococcus faecium*. The culture media used was Lactobacilli MRS broth for *B. cereus* and *E. faecium* and nutrient broth for *P. aeruginosa*.

Activation and inoculation of the microorganisms

The microorganisms were activated in 10 mL of culture media and then incubated at 36°C for 12 h. After this period, the cultures were subcultured in the same media and incubated at 35°C for 10 h. After activation, 400 mL of sterilized milk was inoculated (121°C/15 min) with *P. aeruginosa*, *B. cereus* or *E. faecium*, alternatively. The cultures were inoculated to obtain approximately 1.0×10^6 CFU/mL. The count of microorganisms was done using a pour-plate technique with standard plate count agar (PCA) to verify the number of bacteria in the milk. In the specific case of *B. cereus*, the plating was made for total cell count and spores count. To count spores, the suspension composed of milk and *B. cereus* was heated at 70°C/30 min with subsequent plating.

Description of equipment and experimental model

To determine the ability of the three bacterial cultures to adhere to steel surfaces, we used a stainless steel milk processing circuit (Figure 1), which constituted a ¾-inch-diameter (internal) pipe with a total length of 5.8 m. The milk circulated through the pipe from a tank of 25 L that served as a reservoir. At specific points in the pipe test coupons of 90° (Elbow), T-coupons and cylindrical-shaped coupons made of a stainless steel were installed. The internal surface area of the test coupons was as follows: 108.06 cm^2 for the T-shaped coupons, 52.74 cm^2 for the 90° (Elbow) coupons and 84.69 cm^2 for the cylindrical coupons.

To allow for adhesion, the stainless steel test coupons were removed from the equipment, filled with inoculated milk and closed with cap. The quantities used were 27 mL of milk in the elbow coupon (1A and 1B), 57 mL in the T (3A and 3B) and 49 mL in the cylindrical coupon (2A and 2B) followed by incubation at 18°C for 12 h. After this period, milk samples from the coupons were plated as described previously and the remainder discarded. Sterile milk was added to the test coupons, maintained inside for two minutes and then discarded to remove planktonic cells and/or spores that had reversibly adhered. The test coupons 1A, 2A and 3A were filled with a 2% sodium citrate solution (20 mL in the elbow coupon, 30 mL in the cylindrical coupon and 40 mL in the T-shaped coupon) and washed with manual agitation for 15 min. The wash solution was then plated to determine the number of bacteria that adhered to the test coupons before milk circulation. The sodium citrate solution was discarded and the three coupons (1A, 2A and 4A) were treated for five minutes with a sodium hypochlorite solution at pH 7.5 containing 300 mg/L of free residual chlorine. The coupons were then washed three times in distilled water and subsequently

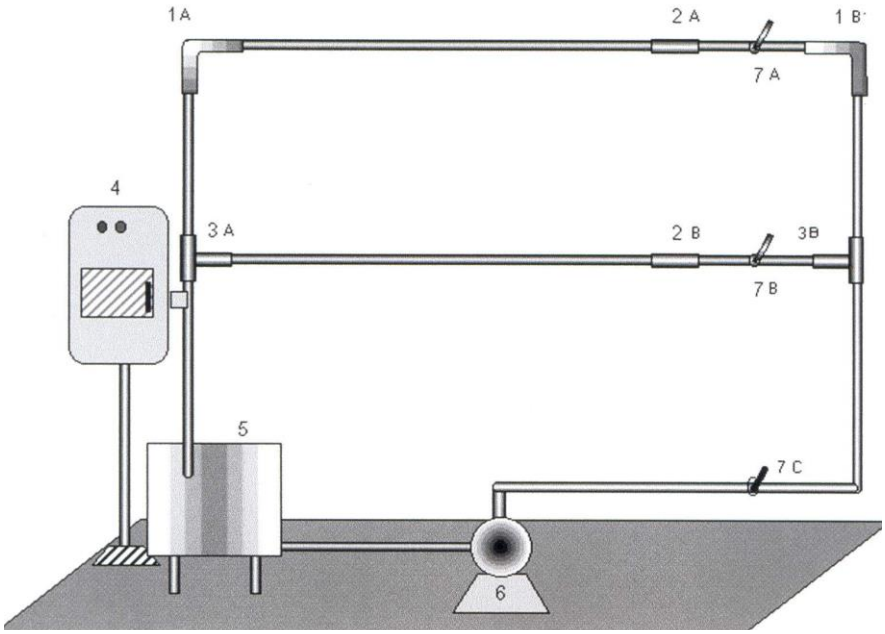

Figure 1. Model of the milk processing line (1 - 90° (Elbow) test coupon; 2 - cylindrical test coupon; 3– T test coupon; 4 – on/off control; 5 - milk reservoir; 6 - centrifugal pump; 7– butterfly valves).

used in the circuit model. The six test coupons were connected to the equipment and 10 L of sterilized milk (121°C for 15 min) was added to the reservoir to circulate for 10 min at 1 m/s at an average temperature of 15°C.

To determine the flow rate at which a velocity of 1.0 m/s was achieved, we used the following calculation:

$$X = \frac{Y\ (m/s)}{\pi\ (R)^2}$$

where: Y = the desired velocity; R = the pipe radius.

The coupons 1B, 2B and 3B, unwashed, were removed from the system and filled with citrate for 2 min with subsequent disposal of the liquid. After that, the coupons were rinsed with a sodium citrate solution as described above. The wash solutions were plated after adequate dilution. Plating, in duplicate, was performed using plate count agar and the incubation was at 35°C for 48 h. The experiment was done in three repetitions.

The experiment was conducted using a split-plot design in which four bacterial forms are used as main treatments and three types of test coupons as secondary treatments. Each experiment was performed in triplicate. Statistical analysis was performed using the number of decimal reductions in the population of microorganisms before (DR_A) and after (DR_B) milk circulation. To determine DR_A, the following calculation was done:

$DR_A = \log (N_0 \times 133) - \log (N_1 \times 245,5)$

Where: N_0 = total number of bacteria (planktonic) inside the coupon after 12 h of incubation; N_1 = number of adhered bacteria inside of the coupon after 12 h of incubation.

The number of sessile cells (N_1) was obtained by washing coupons 1A, 2A and 3A and plating 1 mL aliquot of sodium citrate solution used to wash the test coupons. The value obtained was multiplied by the total wash volume used in the coupon. To obtain N_2, coupons 1B, 2B and 3B were also washed after milk circulation at the desired velocity. To determine DR_B, the following calculation was done:

$DR_B = \log N_1 - \log N_2$

where: N_2 = number of bacteria that remained adherent to the coupon after milk circulation. Therefore, adhesion was considered to be greater for bacteria that had a lower decimal reduction. For comparisons of interest, we performed a Tukey test at 5% probability ($P<0.05$).

RESULTS AND DISCUSSION

The data showed that among the bacteria evaluated, *E. faecium* had the greatest ability to multiply at 18°C in milk (Table 1). We observed that this microorganism increased by about 2 logarithmic cycles in 12 h. *P. aeruginosa* increased by 0.9 logarithmic cycles, whereas *B. cereus* (spores and vegetative cells) increased by 0.4 logarithmic cycles.

We observed a small increase in the number of *B. cereus* spores from 7.8×10^2 CFU/mL at the moment of inoculation to 1.4×10^3 CFU/mL after 12 h.

If we consider that the total area of the three coupons was 245.5 cm^2 and the number of *P. aeruginosa* before the milk circulation was 2.4×10^5 CFU/cm^2 (Table 2), we will come to the conclusion that the total number of adhered cells was 5.9×10^7 CFU. Considering also that there was 7.3×10^6 CFU/ml of milk in the group of coupons which had a total volume of 133 ml milk, we conclude that total number of bacteria in milk at 12 h was

Table 1. Number of colony forming units (CFU/mL) in milk immediately after inoculation and after 12 h of incubation at 18°C. Average of three replicates.

Time	*Enterococcus faecium*	*Pseudomonas aeruginosa*	*Bacillus cereus (spores + vegetative*	*Bacillus cereus* (spores)
	CFU/mL			
Initial	2.4×10^6	9.3×10^5	1.2×10^6	7.8×10^2
12 h (N_0)	2.1×10^8	7.3×10^6	3.0×10^6	1.4×10^3

Table 2. Number of adherent cells in the pipe (test coupons) before and after the circulation of milk at 1 m/s for 10 min.

Adhesion	*Enterococcus faecium*	*Pseudomonas aeruginosa*	*Bacillus cereus (esporos + vegetativas*	*Bacillus cereus* (esporos)
	CFU/cm^2			
Before milk circulation (N_1)	6.5×10^5	2.4×10^5	3.9×10^4	1.9×10^2
After milk circulation (N_2)	3.3×10^4	1.7×10^4	9.0×10^2	7.8×10^0

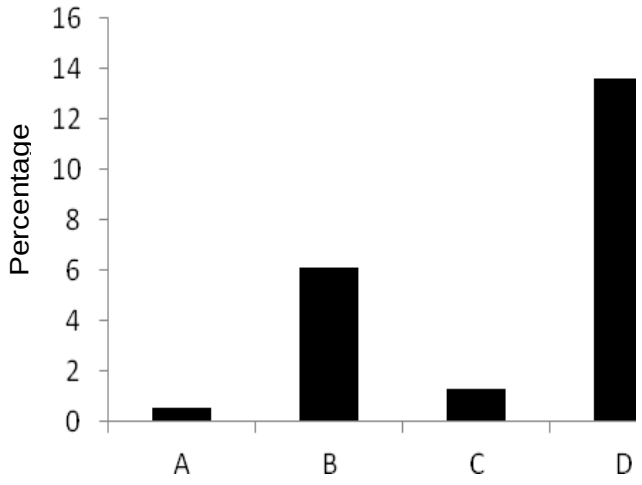

Figure 2. Percent adhesion of bacteria in coupons, before the milk flow. Calculated in relation to number of bacteria (CFU/mL) in the coupon after 12 h in stainless steel at 18°C and number of adherent cells (N_1). *Enterococcus faecium* (A), *Pseudomonas aeruginosa* (B), *Bacillus cereus* vegetative cells and spores (C) and (D) spores of *B. cereus.*

Table 3. Decimal reductions of adhered cells in relation to planktonics microorganisms in test coupons upon 12 h (DR_A= Log N_0 x 133 – Log N_1 x 245,5) of incubation at 18°C.

Microorganisms	DR_A
Bacillus cereus (spores)	0.62^A
Pseudomonas aeruginosa	1.22^B
Bacillus cereus (spores and vegetative)	1.69^C
Enterococcus faecium	2.24^D

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

9.7 x10^8 CFU; thus 6.0% of these bacteria were adhered.

When evaluating bacterial adhesion of *E. faecium* after 12 h (6.5×10^5 CFU/cm^2), in relation to the planktonics cells in milk (2.1×10^8 CFU/mL), we observed 0.31% (Figure 2) of the adhered cells; *B. cereus* vegetative and spore forms 1.28% and spores of *B. cereus* (13.6%). This result is consistent with studies of Suarez (1991); these studies observed that, in several species of psychrotrophic microorganisms isolated from milk, Gram negative species had an increased ability to adhere to stainless steel, rubber and glass surfaces than Gram positive species.

It is interesting to note the high percentage of adhesion obtained with spores, which reached 13.6%, about 10 times higher than the adhesion of vegetative cells and spores. According Ronner et al (1990), some spores are highly hydrophobic which facilitates their adhesion to surfaces. In a study involving five species of bacteria that produce spores, it was observed that the spore of *B. cereus* presented the greater adhesion capacity, about 45% in hydrophobic surface, whereas *Bacillus licheniformis* has better adhesion on hydrophilic surface. The study also showed that the adhesion ability of the spore form is much higher than vegetative form of the same microorganism.

In Table 3, it is shown that *B. cereus* showed the largest adhesion capacity, in other words, the lowest decimal reduction. These spores likely adhere to surface of equipment then germinate and compromise the quality of the milk. The following are ranked in ascending order according to their decimal reduction: *P. aeruginosa, B. cereus*, including spores and vegetative cells, and *E. faecium*. Based on analysis of variance of the decimal reduction and Tukey test, it is concluded that there is a significant difference ($p<0.05$) between the types of bacteria used in relation to the ability of adhesion.

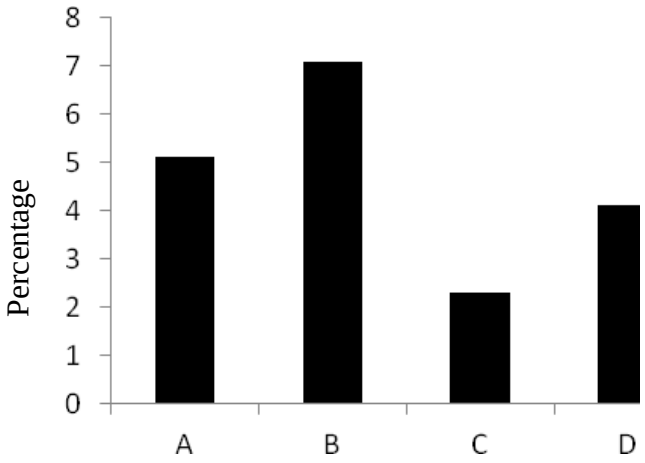

Figure 3. Percent adhesion of bacteria in coupons, after milk circulation. Calculated in relation to number of bacteria adhered before milk circulation (N_1) and after circulation (N_2). *Enterococcus faecium* (A), *Pseudomonas aeruginosa* (B), *Bacillus cereus* vegetative cells and spores (C) and (D) spores of *B. cereus*

Table 4. Decimal reductions (DR_A= Log N_0 – Log N_1) of adhered cells after milk circulation model at a velocity of 1 m/s for 10 min at 15°C.

Microorganisms	DR_A
Pseudomonas aeruginosa	1.15^A
Enterococcus faecium	1.28^A
Bacillus cereus (spores)	1.39^A
Bacillus cereus (spores and vegetative)	1.63^A

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

After milk circulation (Figure 3), we observed that 5% of *E. faecium* cells remained adhered. We calculated 6.5 x 10^5 CFU/cm^2 of *E. faecium* before milk circulation, where this number was reduced to 3.3 x 10^4 CFU/cm^2 after circulation. Our results also indicated that 7.1% of the *P. aeruginosa* cells remained adherent in the circuit model. This percentage, calculated based on the number of adherent cells before milk circulation, represented 1.7 x 10^4 CFU/cm^2 of the surface. The number of microorganisms here is still large enough to cause milk contamination because the lipases produced by *Pseudomonas* sp. are extremely resistant to thermal heat treatment at 110°C for 10 min (Robinson, 1990).

Our results also indicated that 7.0% of the *P. aeruginosa* cells remained adherent in the circuit model. The number of microorganisms here is still large enough to cause milk contamination because the lipases produced by *Pseudomonas* sp. are extremely resistant to thermal heat treatment at 110°C for 10 min (Robinson, 1990).

There was 1.28% adhesion of *B. cereus* spores and vegetative cells before milk circulation, and 2.4% of the cells that adhered resisted the milk flow. However, it should be noted that although the spores had an increased ability to adhere to surfaces (13.6%) in foods, 4.1% of the adhered spores resisted the flow of milk. This may be because spores do not have the ability to produce polymeric substances that facilitate their adhesion to coupons. Consequently, the spores only remain adherent by forces, such as electrostatic attraction, which reduces their counts after milk circulation to levels well below the initial counts.

The decimal reduction, for different microorganisms after milk circulation is shown in Table 4. The analysis of variance (Table 5) performed for the results showed that there were no significant differences ($p \geq 0.05$) in adhesion between the different types of bacteria. However, there was a difference with respect to the removal of cells from the different types of coupons.

The interaction of microorganisms with the different coupons was not significant (Table 5). This analysis was intended to determine whether a particular bacterium adhered at a higher percentage to one particular type of coupon, whereas another species could have a higher percentage of adhesion to a second type of coupon.

The highest rate of bacterial removal occurred in the T-shaped coupon (Figure 4), whereas the lowest rate of removal occurred in the cylindrical coupon. This difference was significant at level of 5% (Table 6). We did not observe a significant difference in Tukey test ($p \geq 0.05$) in the removal of bacteria from the cylindrical and elbow or T and elbow coupons.

It is possible that the turbulence in cylindrical pipes is lower than in pipes with contouring formats, such as in elbow and T-shaped pipes. Therefore, the shear caused by the fluid on the walls of the cylindrical test coupons is lower, and this can hamper the removal of micro-organisms from surfaces.

Conclusion

We observed a significant difference ($p<0.05$) in the ability of the three microorganisms to adhere to stainless steel before milk circulation. Thus, 13.6% of *B. cereus* spores adhered, the vegetative and spore forms of *B. cereus* together showed 1.28% of adhesion, *P. aeruginosa* and *E. faecium* had 6.0 and 0.31% respectively. This demonstrates that *B. cereus* spores have an increased adhesion ability as compared to the other species evaluated.

After milk circulation, there were no significant differences in the number of bacteria that remained adherent ($p \geq 0.05$). However, there were differences in the removal of adherent cells between different types of coupons test. The removal was higher in T coupon and the coupon lower cylindrical presenting statistically

Table 5. Summary of the analysis of variance of decimal reductions in the different microorganisms on the different test coupons after using the milk circulation model at a velocity of 1 m/s for 10 min at 15°C.

S.V.	D.F.	S.S.	M.S.	F	$F_{5\%}$
Bacterium	3	0.7837613	0.2612537	2.2119ns	4.07
residue (a)	8	0.9448708	0.1181088		
Coupon	2	0.4470955	0.2235478	6.30*	3.63
Coupon*inoculation	6	0.4788803	0.07981338	2.25ns	2.74
Residue (b)	16	0.5674057	0.03546286		
TOTAL	35	3.222013			

*Significant at 5% significance by the F-test; [ns] Not significant at 5% significance by the F-test.

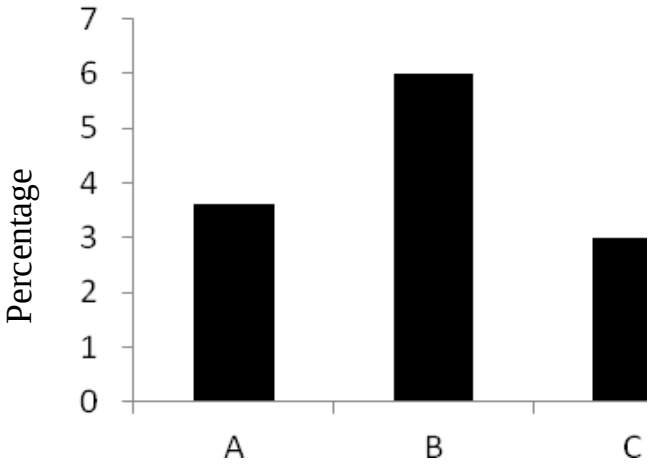

Figure 4. Percent adhesion of bacteria in coupons, after milk circulation. Elbow coupon (A), cylindrical coupon (B), T-coupon (C).

Table 6. Averages of decimal reductions in the microbial populations for the different test coupons after milk circulation at 1 m/s for 10 minutes at 15°C.

Coupon type	DR^B
T	1.5496^A
Elbow	1.4713^{AB}
Cylindrical	1.2840^B

*Averages followed by the same letter do not differ significantly at Tukey test (5%).

significant difference ($p<0.05$). We concluded that it is necessary to avoid T-shaped points in the pipes through which foods pose a barrier to bacterial adhesion.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

Fundação de Amparo e Desenvolvimento da Pesquisa/Universidade Federal do Pará/PA/Brazil.

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academicJournals

Vol. 8(35), pp. 3271-3275, 27 August, 2014
DOI: 10.5897/AJMR2014.6998
Article Number: E3B97B947158
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibiotic resistance trend of *Staphylococcus aureus* isolated between 2010 and 2012 from mastitis cases in Azawak zebu in Niger

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Received 7 July, 2014; Accepted 22 August, 2014

The present study was conducted at the Sahelian Experimental Station in Toukounous Niger, on three herds of Azawak zebu breed in order to evaluate antimicrobial susceptibility of a total of 43 *Staphylococcus aureus* isolated from 164 milk samples of cows with subclinical mastitis from 2010 to 2012. The highest frequency of resistance was observed for the β-lactam family: penicillin (46%) followed by oxacillin (16%). Twenty isolates were sensitive to all tested antibiotics, 12 were resistant to one of them and 11 were multi-resistant (2 to 5 antibiotics). The resistance percentage to oxacillin, enrofloxacin and clindamycin varied significantly over the 3 years (p <0.05) but not to the other antibiotics. Moreover, 90% of *S. aureus* isolates resistant to penicillin detected by *in vitro* disk diffusion possessed the *blaZ* gene. In conclusion, the isolates from cases of mastitis at Toukounous are more resistant to the antibiotics frequently used for treatments at the station than to other antibiotics.

Key words: Mastitis, *Staphylococcus aureus*, antibiotic resistance, Azawak zebu, Niger.

INTRODUCTION

Mastitis is the inflammation of the mammary gland most frequently caused by bacterial infection. Bacterial mastitis is a disease causing considerable economic loss in cattle farming. Milk deficits during mastitis vary from 25 to 48% depending on the intensity of inflammation and the stage of lactation (Gebreyohannes et al., 2009). Treatment (use of antibiotics) costs can be expensive and the incurable cows must be reformed, a preventive method truly efficient

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but also very expensive (Radostits et al., 2007). At the Sahelian station in Toukounous (Niger) reformed cows are sold to private farmers for fattening before slaughter.

Several bacterial species can cause bovine mastitis (Radostits et al., 2007). In many countries including Niger, *Staphylococcus aureus* is one of the most frequently isolated species from subclinical and clinical mastitis (Pradeep et al., 2003; Bada et al., 2005; Harouna et al., 2009; Issa et al., 2013). As a consequence of the wide use of antibiotics in dairy cattle farms, several mastitis-causing bacterial species have become resistant to the most frequently used antibiotics (Werckenthin et al., 2001). Nevertheless, antimicrobial therapy still plays an important role in mastitis control by reducing the infection levels and preventing new cases (Rabello et al., 2005).

In Niger, zebu cattle of the Azawak breed offer the best dairy aptitude and since 1975 the Niger government has opted for a policy of development and improvement of the breed. The experimental station in Toukounous was therefore set up with the objective of improving the dairy production by genetic selection and of promoting its distribution to individual farmers across the whole country (MRA, 2002). But, according to the available data in the yearly reports, the extensive use of antibiotics of the β-lactam (amoxicillin, ampicillin), tetracyclin (tetracyclin, oxytetracylin), aminoglycoside (streptomycin, gentamicin) and sulfonamide families is noticeable, more especially for the treatment of intestinal and mammary gland infections (SSET, 2009). However, the influence of this wide antibiotic use on the level of resistance of mastitis-causing bacterial species is unknown. It is therefore important to study not only the prevalence of the different antibiotic resistance profiles of the most frequent mastitis-causing bacterial species, but also their evolution in time to foresee the efficiency of the treatments in the future.

Therefore the objective of this survey was to follow the evolution of antibiotic resistance of the *S. aureus* isolates during a three year period of time (2010-2012) from cases of mastitis in Azawak zebu at the Sahelian experimental station of Toukounous, Niger.

MATERIALS AND METHODS

Bacterial isolation and identification

A total of 164 milk samples were collected between 2010 and 2012 from cows of the three Azawak zebu herds (elites, non-elites and primiparous) of the Toukounous station with subclinical mastitis. The bacteriological analysis of milk samples and the identification of *S. aureus* were performed as described previously (Issa et al., 2013).

Antibiotic susceptibility testing and resistance profiles

The susceptibility of the *S. aureus* isolates to seven antibiotics was determined by the disc diffusion method on Mueller-Hinton agar plates (Becton Dickinson, Belgium) as described by Bauer et al. (1966). Isolates were tested with discs with tetracyclin (30 UI), penicillin G (10 UI), gentamicin (10 UI), trimethoprim-sulfamethoxazole (1.25 µg / 23.75 µg), enrofloxacin (5 µg), clindamycin (2 UI) and oxacillin (5 µg) (Becton Dickinson, Belgium). The diameter in mm of the inhibition zone of each tested antibiotic was compared with the recommendations of the French Committee Guidelines for susceptibility testing (Comité de l'Antibiogramme vétérinaire de la Société Française de Microbiologie, 2010) to determine the susceptibility/resistance profile of each isolate. The data were processed in a spreadsheet program (Excel for Windows, Microsoft).

PCR characterization

All the isolates were tested for the presence of *blaZ* gene (coding for β-lactamase) by polymerase chain reaction (PCR) with forward primer 5'-TAA GAG ATT TGC CTA TGC TT-3' and reverse primer 5'-TTA AAG TCT TAC CGA AAG CAG-3' (Olsen et al., 2006). DNA extraction was carried out using the ChargeSwitch DNA Mini Bacteria Kit (Invitrogen, USA) according to the manufacturer's instructions for staphylococci. The PCR reaction mixture contained: 1U of *Taq* DNA polymerase (New England Biolabs, USA), 2.5 µl of 2 mM deoxynucleoside triphosphates (Eurogentec, Belgium), 2.5 µl of 10X ThermoPol Reaction Buffer, 1 µl of each primer (10 µM) and 1.5 µl of a DNA template in a total volume of 25 µl. The PCR reactions were performed using a Mastercycler® (Eppendorf, France). The PCR-amplified DNA fragment of 519 bp was isolated by electrophoresis in a 1.5% agarose gel and photographed under UV light after ethidium bromide staining.

Statistical analysis

To compare the evolution of the percentages of resistant *S. aureus* to the different antibiotics over the years (2010, 2011 and 2012), the Fisher's Exact Test was performed (SAS, 2001). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

A total of 43 *S. aureus* isolates were recovered from the 164 milk samples: 22 isolates in 2010, 9 in 2011 and 12 in 2012, with 7 isolates from primiparous cows, 21 from elite cows and 15 from non-elites cows (Table 1). Twenty isolates (46%) were sensitive to all antibiotics tested, but 23 *S. aureus* isolates (54%) were resistant to at least one antibiotic tested: 14 in 2010 (33%), 5 in 2011 (12%) and 4 in 2012 (9%) ($p<0.05$).

The average resistance rates over the three years differ according to the antibiotic. As many as 20 of the 43 *S. aureus* isolates (46%) were resistant to penicillin (Figure 1); much less isolates were resistant to oxacillin (16%), gentamicin (9%), trimethoprim-sulfamethoxazole (9%), tetracyclin (7%), clindamycin (5%) and enrofloxacin (2%). The annual resistance rates in 2010 (22 isolates), 2011 (9 isolates) and 2012 (12 isolates) were the following : penicillin (64, 22 and 33%), oxacillin (14, 44 and 0%), gentamicin (4, 0 and 0%), trimethoprim-sulfamethoxazole (14, 11 and 0%) tetracyclin (14, 0 and 0%), clindamycin (4, 11 and 0%) and enrofloxacin (0, 0 and 8%). The Fisher's Exact Test confirmed a significant difference ($p<0.05$) in the resistance percentages to oxacillin, enrofloxacin and clindamycin over the 3 years but not to the other antibiotics. All but three of the 23 resistant

Table 1. Number of *S. aureus* isolated at Toukounous according to the heard and to the year.

Herd	2010	2011	2012	
	No of sampled animal / No of isolated *S. aureus*			Total
Elite	25/9	19/4	32/8	76/21
Non-elite	20/7	24/4	19/4	63/15
Primiparous	19/6	6/1	0/0	25/7
Total	64/22	49/9	51/12	164/43

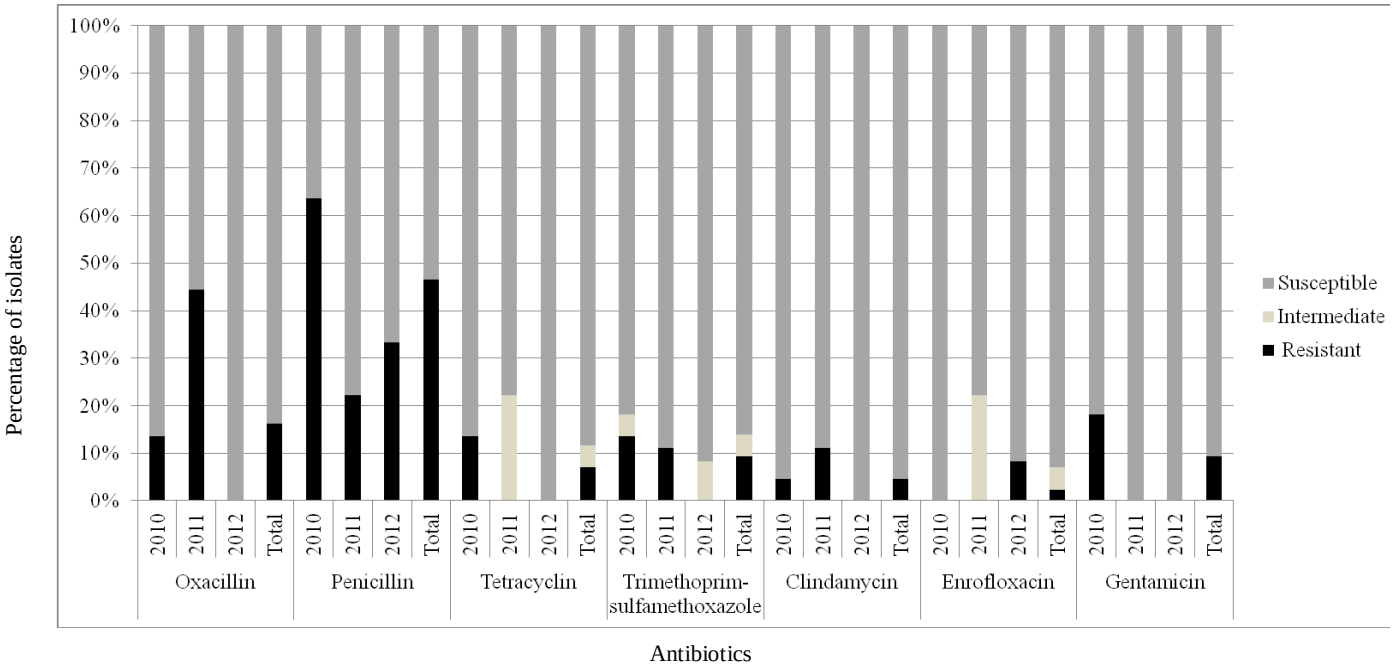

Figure 1. Antibiotic resistance profiles of the 43 *S. aureus* isolates in 2010, 2011 and 2012.

isolates were resistant to penicillin and 11 of them were multidrug resistant (Table 2): six isolates were resistant to two antibiotics, four to three antibiotics and one to 5 antibiotics. These results are probably related to the frequent use of β-lactams in the treatment of any suspected bacterial infection at the station followed by tetracyclins alone or in combination with aminoglycosides in second intention.

The average resistance rates in Toukounous are comparable, sometimes lower, to those obtained in previous studies performed in different African countries and in Pakistan. For instance, 64, 56, 54 and 100% of the *S. aureus* isolates respectively in Tunisia, Pakistan, Senegal and Uganda, are resistant to penicillin (Ben Hassen et al., 2002; Arshad et al., 2006; Kadja et al., 2010; Kateete et al., 2013). The results are also similar for oxacillin and tetracyclin. Oxacillin resistance rate is 7.6, 10 and 15% respectively in Nigeria, Senegal and Morocco (Bendahou et al., 2008; Kadja et al., 2010; Suleiman et al., 2012); whereas tetracyclin resistance rate is 13 and 15% in two previous studies in Niger in 2007 and 2009 (Harouna et al., 2009; Issa et al., 2013). Finally, 25% of the isolates of this study are multiresistant (resistance to at least two antibiotics), a result also comparable to the 23% reported by Zanette et al. (2010), but lower than the 62% reported by Meideiros et al. (2009), both in Brazil.

Thirteen cows could be followed between 2010 and 2012 to assess the individual antibiotic resistance evolution (Table 3). The antibiotic resistance profiles of the *S. aureus* isolated from the same cow differed from one year to another suggesting that the isolates are not clonal and that reinfection of the udder occurred during the three years. The Pulsed Field Gel Electrophoresis profiles (manuscript in preparation) confirm the hypothesis that isolates from the same cow are indeed not clonal.

Of the 20 isolates of *S. aureus* resistant to the penicillin

Table 2. Resistance profiles of the 23 *S. aureus* isolates resistant to at least one antibiotic.

No. of antimicrobials	Resistance profile					No. of isolates
1	Pen					9
	Oxa					2
	Clin					1
2	Pen	Oxa				2
	Pen	Gen				2
	Pen	Tet				1
	Pen	Eno				1
3	Pen	Oxa	Tet			1
	Pen	Oxa	Tsu			1
	Pen	Tet	Tsu			1
	Pen	Tsu	Gen			1
5	Pen	Oxa	Gen	Tsu	Clin	1
Total						23

Pen = Penicillin, Oxa = oxacillin, Tet = tetracyclin, Tsu = trimethoprim-sulfamethoxazole, Enro = enrofloxacin, Clin = clindamycin, Gen = gentamicin.

Table 3. Antibiotic resistance profiles of the *S. aureus* isolates from 13 cows over three years (2010 to 2012).

Cow identification/ year of isolation	Antibiotic resistance profile		
	2010	2011	2012
56/11	Pen, Oxa, Tsu, Clin, Gen	S	0
61/11	0	Pen, Oxa, Tsu	Pen
63/11	0	Clin	S
65/11and 68/11	S	0	S
66/11	S	Oxa	0
70/11	Pen, Gen	0	S
71/11	Pen	0	S
76/11	Pen, Tet, Tsu	0	S
79/11	S	S	S
86/11	Pen	Pen, Oxa	Pen, Enro
94/11	0	0	Oxa
96/11	S	Oxa	S

Pen = penicillin, Oxa = oxacillin, Tet = tetracyclin, Tsu = trimethoprim-sulfamethoxazole, Enro = enrofloxacin, Clin = clindamycin, Gen= gentamicin, S = sensitive to all tested antimicrobials, 0 = no *S. aureus* isolated from milk sample.

by the *in vitro* disc diffusion test, 18 (90%) tested positive by PCR for the *blaZ* gene suggesting that the production of β-lactamase by most penicillin-resistant *S. aureus* of this study is encoded by the *blaZ* gene (Zscheck and Murray, 1993).

In conclusion, our study provides recent data on the distribution of antimicrobial resistance at the pilot farm of Toukounous. The massive resort to β-lactam antibiotics to treat any condition is most probably responsible for the high rate of resistance observed at the farm. Besides the resistance problem, it has also been reported that intra-mammary infection remained significantly more often chronic if it was caused by *blaZ*-positive than *blaZ*-negative isolates of *S. aureus* (Taponen et al., 2003). It is therefore an absolute necessity to be more cautious and strict in the application of treatments with antibiotics, particularly in the use of the β-lactams.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Abdoulkarim Issa Ibrahim is a PhD student at the University of Liège (Belgium), and is supported financially by the Belgium Technical Cooperation (CTB). We are also grateful to the staff of station in Toukounous and especially to Dr Chanono Mogueza, the manager.

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academicJournals

Vol. 8(35), pp. 3276-3283, 27 August, 2014
DOI: 10.5897/AJMR2014.6844
Article Number: 4F5229947160
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Non-invasive detection of *Helicobacter pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants

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Received 21 April, 2014; Accepted 4 August, 2014

Helicobacter pylori **is a microaerophilic spiral-shaped Gram-negative bacterium that infects approximately 50% of the world's population, particularly in developing countries. Infections early in childhood are postulated to induce a low-grade chronic inflammatory condition. This study aimed to determine the prevalence of *H. pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants and to define the possible infection associated risk factors. Non invasive test using polymerase chain reaction on stool samples was used for detection of these genes. Prevalence of *H. pylori* among those infants was 88.9%. Prevalence of *ureA, vacA, cagA* and *babA2* was 86.9, 98.8, 71.4 and 67.8%, respectively. Risk factors significantly associated with infection included bed sharing, premastication of food and nursery attendance (P<0.005). The prevalence of *H. pylori* infection among Egyptian infants is very high with high prevalence of virulence genotypes, so follow up of these infants and repetition of this study on a wider scale is recommended.**

Key words: *Helicobacter pylori*, virulence, Egypt, infants.

INTRODUCTION

Helicobacter pylori (H. pylori) is a microaerophilic motile Gram-negative spiral-shaped bacterium (Wen and Moss, 2009). Approximately, 50% of the world's population is infected with the organism (Torres et al., 2000). Poor socio-economic conditions were found to be associated with early colonization in children. High prevalence of *H. pylori* among people in low-income countries has been demonstrated by several studies (Hestvik et al., 2010). Socio-economic status includes not only income but also living conditions, sanitation and educational level (Khalifa et al., 2010). Fayoum Governorate, where this study was based, is a low socioeconomic area located in Upper Egypt. It is surrounded by many villages with limited services especially in the health sector. Total population of Fayoum Governorate is about 3 millions.

H. pylori causes chronic infection of the stomach for almost the entire lifetime of the individual. Chronic gastritis and peptic ulcers or gastric carcinoma can be associated

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with chronic *H. pylori* infection (Frenck and Clemens, 2003). These long term complications associated with *H. pylori* infection varies between developed and developing countries. The age at which an individual is infected with *H. pylori* may play a role in this difference (Khalifa et al., 2010).

Early infections in childhood are postulated to induce a low-grade chronic inflammatory condition which may develop into pre-malignant changes and eventually gastric carcinoma. In contrast, when infection is acquired later in life, it is more likely to induce a brief inflammatory response. Although, the prevalence of *H. pylori* infection in Egyptian population is very high and acquired early in life, the prevalence of gastric cancer in Egypt is very low ($3.4/10^5$) (Hussein, 2010).

Of those infected, disease development is influenced by *H. pylori* strain virulence. Bacterial virulence is the ability of some bacteria to cause disease. The major *H. pylori* virulence factors include the vacuolating toxin VacA, the blood group antigen binding adhesin (BabA) and the cag pathogenicity island (cagPAI). The cagPAI is a cluster of genes with seven of these genes, *virB4, virB7, virB8, virB9, virB10, virB11*, and *virD4*, homologous to the gene components of the type IV secretion system (T4SS) of *Agrobacterium tumefaciens* (Censini et al., 1996).

The cytotoxin associated gene A (cagA), being a marker for the presence of the cag pathogenicity island (cagPAI), was the first recognized virulence gene in the *H. pylori* genome. It is present in 60-70% of *H. pylori* strains and encodes a high molecular weight antigenic protein (120-140 kDa) (Atherton, 1998; Queiroz et al., 2000).

Vacuolating cytotoxin, VacA, induces cytoplasmic vacuolation in cultured epithelial cells. Unlike the cagPAI, the *vacA* gene is present in almost all strains. The vacuolating cytotoxin A (*vacA*) gene exists in different subtypes, varying in the signal (*s1 or s2*), the intermediate (i) and the middle (*m1 or m2*) regions. Polymorphisms among the VacA alleles result in different levels of cytotoxicity. All possible combinations from these regions have been identified and among them, vacuolating activity is highest in *s1m1* strains, less in *s1m2* strains and is absent in *H. pylori* expressing *s2m2* forms (Ko et al., 2008).

The blood group antigen binding adhesin (BabA), encoded by the *babA2* gene, binds to Lewis b antigens and ABO antigen. There are two distinct *babA* alleles (babA1 and babA2). Only the *babA2* allele is functionally active (Ilver et al., 1998).

The combined presence of *babA, cagA* and *vacAs1* ‘‘triple-positive strains”, was reported to be associated with duodenal ulcer and gastric adenocarcinoma in Western populations (Zambon et al., 2003).

Virulence-associated gene, *ureA,* is one of the genes of the urease operon encoding the urease subunit A (Blom et al., 2000). The *ureA* is widely used for identifying *H. pylori* by PCR (Clayton et al., 1992; Espinoza et al., 2011). Other targets of PCR amplification methods include the 26-kDa species-specific antigen (*SSA*) gene and the phosphosamine mutase (*glmM*) gene (Smith et al., 2004). Other noninvasive *H. pylori* diagnostic tests include: culture, the ^{13}C urea breath test, antigen enzyme immunoassay (EIA) for detection of *H. pylori* in faeces and ELISA serology (Monteiro et al., 2001).

This study aimed to determine the prevalence of *H. pylori* virulence genotypes *ureA, vacA, cagA* and *babA2* among asymptomatic Egyptian infants using stool samples as a non invasive screening test. Determination of the possible infection associated risk factors was also an objective of the study.

MATERIALS AND METHODS

This is a cross-sectional study of virulent *H. pylori* prevalence in asymptomatic Egyptian infants in Fayoum Governorate, a low socio-economic governorate. Virulence genes of *H. pylori* were detected by polymerase chain reaction of stool samples, as a non invasive test.

Egyptian infants included in the study should fulfilled the following criteria (a) aged ≤24months (b) asymptomatic regarding gastro-intestinal (GIT) symptoms (c) no antibiotics received within the 2 months before stool collections.

One hundred and eighty nine infants, with an average age (16.1 m±5.6) were included in the study. This cohort included 126 males (66.66%) and 63 females (33.33%). Before sample collection, verbal consent was taken from the parents. A detailed history and physical examination was done for each infant included in the study.

Specimen collection and DNA extraction

Stool, as a noninvasive sample, was used for screening. Stool samples were kept in buffered phosphate saline (BPS) at -20°C until DNA extraction to keep DNA intact as long as possible. DNA was extracted from stool samples by the EZ-10 Spin Column Soil, fecal samples DNA Mini-Preps Kit (Biobasic, Canada) following the manufacturer’s instructions. Extracted DNA was stored at -20°C until use.

The *ureA, cagA, vacA and babA2* genotyping

Stool samples were collected from 189 asymptomatic infants. Detection of *H. pylori* disease-causing genotypes was performed on all samples by PCR using 5 primer sets to amplify *ureA, cagA,* vacA and babA2 specific bands. Oligonucleotide primers used in the amplifications were obtained from Biobasic, Canada. In all cases, separate PCR reactions were performed for detection of each gene. A final reaction volume of 25 µl was used. Reaction mixture contained 12.5 uL, 2X ready to use PCR master mix (PCR-EZ D-PCR Master Mix, Biobasic, Canada) consisted of 1 Unit of Taq Polymerase, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris HCl (pH 8.75), 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mM $MgCl_2$ and 200 mM dNTPs. Also, 0.6 mM sense and antisense primers, 100 ng genomic DNA and a PCR grade water was added upto the final reaction volume. Used primers and its cycling conditions are shown in Table 1.

Statistical analysis

Collected data were computerized and analyzed using Statistical Package for Social Science (SPSS) version 16. Descriptive statistics

Table 1. Oligonucleotide primers used for the amplification of *ureA, cagA, vacA and babA2* genes and its cycling conditions.

Primer designation	Sequence (5'-3')	PCR product size (bp)	Cycle Conditions*	Reference
ureA F ureA R	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	(35 cycles) 94°C, 1 min; 45°C, 1 min; 72°C, 1 min	Clayton et al. (1992)
cagA F cagA R	GATAACAGGCAAGCT TTTGA CTGCAAAAGATTGTTTGGCAGA	349	(35 cycles) 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1 min,	Atherton et al. (1995)
vacAs F vacAs R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAA	s1-259 s2-286	(35 cycles) 94℃ for 1 min, 52℃ for 1 min and 72℃ for 1 min,	Atherton et al. (1995)
vacAm F vacAm R	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	m1-567 m2-642	(40 cycles) 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min,	Torres et al. (2009)
babA2 F babA2 R	AATCCAAAAAGGAGAAAAAGTATGAAA TGTTAGTGATTTCGGTGTAGGACA	832	(40 cycles) 94℃for 1 min, 60℃ for 1 min and 72℃for 1 min,	Mizushima et al. (2001)

*The PCR had an initial step at 94℃ for 1 min, a final extension at 72℃ for 5 min. A thermal Cycler (Bio-Rad, USA) was used. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide.

Table 2. Prevalence of *H. pylori* according to age groups (<12 m and ≥12m).

Case	Age (months)		Total	P value
	<12 m	≥12 m		
Negative	2 (3.8%)	19 (11.9%)	21 (11.1%)	0.162
Positive	50 (96.2%)	118 (88.1%)	168 (88.9%)	
Total	52 (100%)	137 (100%)	189 (100%)	

were used to describe variables; percent, proportion for qualitative variables. Mean, standard deviation and range were used to describe quantitative variables. Chi-square test was done for comparing qualitative variables between groups. P values of less than 5% were considered statistically significant.

RESULTS

The study included 189 asymptomatic Egyptian infants. Sixty three were females and 126 were males (females: males = 1:2). Age range was 6-24 months (16.1 m±5.6). Overall prevalence of *H. pylori* was 88.9% (168/189). No significant difference in prevalence between boys and girls was found (P= 0.999). We examined the effect of age on *H. pylori* infection by dividing the children into 2 groups: those <12 months, and ≥12 months. We found no significant difference in the prevalence between these age groups (P= 0.162). Data are shown in Table 2.

Risk factors assessment

Bed sharing, premastication of food and nursery attendance were found to be significantly associated with *H. pylori* infection (P<0.005). Breast feeding didn't appear to be protective, but bottle feeding and usage of cow milk as a feeding supplement appeared to be significantly associated with infection (P=0.01). No significant association was observed with order between siblings, number of siblings, bath sharing, sewage disposal and clean water supply (P>0.05). A summary of results pertaining risk factors positively associated with infection is presented in Table 3.

Prevalence of *ureA, cagA, vacA and babA2* in positive samples

The prevalence of *H. pylori* in the stool samples tested was 88.9% (168/189) of which 41.7% were triple positive *"cagA, vacAs1 and babA2"*. The prevalence of virulence genes *ureA, cagA, vacA* and *babA2* in the positive stool samples for *H. pylori* is presented in Table 4.

Genes were identified according to sizes of PCR product bands in agarose gel. The ureA gene, visualized as 411 bp band, was detected in 86.9% of cases. Amplified *cagA* gene was visualized as a band of 349 bp in 71.4% of the positive strains and *vacA* subtypes were detected in 98.8% of strains. The *babA2* gene was visualized as a band of 832 bp in 67.8% of the positive strains.

The most virulent *vacAs1* genotype was the predominant

Table 3. A summary of results pertaining risk factors positively associated with infection.

Parameter	Positive		Negative		P value	Odds ratio or (CI 95%)
	Yes	No	Yes	No		
Bed sharing	106 (56.1%)	62 (32.8%)	3 (1.6%)	18 (9.5%)	0.000	13.68 (1.19-1.4)
Nursery attendance	84 (44.45%)	84(44.45%)	0	21 (11.1%)	0.000	2.0 (1.72-2.33)
Premastication of food	38 (20.1%)	130 (68.8%)	0	21 (11.1%)	0.000	1.29 (1.19-1.4)
Breast feeding	98(51.9%)	73 (38.6%)	8(4.2%)	10(5.3%)	0.000	1.4 (1.2-1.6)

Table 4. Prevalence of disease causing *H. pylori* genotypes.

Parameter	Positive	Negative	Total
ureA	146 (86.9%)	22 (13.1%)	168 (100%)
vacA	166 (98.8%)	2 (1.2%)	168 (100%)
cagA	120 (71.4%)	48 (28.6%)	168 (100%)
babA2	114 (67.8%)	54 (32.2%)	168 (100%)

Table 5. Relationship between *vacA* subtypes, *cagA, ureA and babA2* genes.

Parameter	*cagA*			*ureA*			*babA2*			
vacA	**positive**	**negative**	**P value**	**positive**	**negative**	**P value**	**positive**	**negative**	**P value**	**Total**
vacAs1m1	38	10	0.047	46	2	0.007	28	20	0.369	48 (28.9%)
vacAs1m2	64	20	0.009	78	6	0.000	68	16	0.000	84 (50.6%)
vacAs2m1	4	0	0.29	4	0	0.29	2	2	0.699	4 (2.4%)
vacAs2m2	18	12	0.24	22	8	0.14	22	8	0.341	30 (18.1%)

Significant association between *ureA* and *cagA* (p=.000) and between *ureA* and *babA2* (p=.002) was found. Relationship between *ureA, cagA and babA2* is shown in Table 6.

Table 6. Relationship between *ureA, cagA and babA2.*

Parameter		*cagA*			*babA2*		
		Positive	**Negative**	**P value**	**Positive**	**Positive**	**P value**
ureA	Positive	114	32	0.00	98	48	0.002
	Negative	6	34		16	24	

genotype in *H. pylori* isolates, and was visualized as a band of 259 bp on agarose gel electrophoresis in 79.5% of positive cases, whereas 20.5% of isolates had the *vacAs2* genotype. The middle *(m2)* region of the *vacA* gene predominated in positive samples (68.7%), while *m2* genotypes were 31.3%. On the other hand, *s1m2* genotype was the most common combination of the *vacA* subtypes in the current study. Prevalence of other *vacA* subtypes and its distribution among *ureA, cagA and babA2* positive and negative cases is summarized in Table 5. A significant association between *ureA, cagA* and *vacAs1m1, vacAs1m2* genotypes was found ($p<0.05$) (Table 5). An association between babA2 and vacAs1m2 was also found (Table 6).

DISCUSSION

To our knowledge, this is the first study to evaluate the prevalence of disease associated virulent *H. pylori* genotypes among asymptomatic Egyptian infants in Fayoum Governorate, a rural low socioeconomic area with restricted economical activities, limited social and medical services. In this study, a non-invasive assay for screening and early detection of *H. pylori* disease-associated genotypes in stool samples was used as recommended by Sicinschi et al (2012), who used PCR for amplification of *H. pylori* virulence genes from stool DNA. The study included 189 infants (63 females and 126 males) aged 16.1 m±5.6 with overall prevalence of 88.9%.

In the current study, no significant difference was found in prevalence between males and females. This supports results obtained by meta-analysis of 10 studies conducted over the last 20 years which found no sex difference regarding prevalence of *H. pylori* among children (de Martel and Parsonnet, 2006). Higher prevalence in males or in females was reported in different other studies (Klein et al., 1994; Ndip et al., 2004; Dube et al., 2009).

An association was found between nursery attendance and *H. pylori* infection in the current study, which can be explained by crowding, lack of proper hygiene and may be mix of infant feeding utensils. Another significant association between *H. pylori* infection and bed sharing was found in this study ($p < 0.05$) and this was in concordance with reports from the developing world; Nairobi, China and Bangladesh (Brown et al., 2002; Langat et al., 2006) as well as the developed world, USA (Staat et al., 1996), as direct transmission through over-crowding was suggested as a mode of transmission of *H. pylori* infection.

A strong association between poverty-related factors and increased risk of acquiring *H. pylori* has been demonstrated by different earlier studies (Malaty et al., 1996; Khalifa et al., 2010). This can explain the high prevalence of infection in the studied community.

Also, pre-mastication of food, which is a habit of many Egyptian mothers, has been found in both Bangladesh and Ethiopia as well as in the current study to be associated with an increased prevalence of *H. pylori* in babies, supporting the possibility of oral-oral transmission (Lindkvist et al., 1998).

Another cause of oral-oral transmission is maternal infection, as an infected mother may play a key role in the transmission of *H. pylori* within the family (Drumm et al., 1990). The high prevalence of *H. pylori* infection in asymptomatic infants can be explained by the high prevalence of *H. pylori* infection in their mothers (Bassily et al., 1999). Bassily et al. (1999), found that 82% of the children born to mothers infected with *H. pylori* also became infected compared to 14% infection in children of non-infected mothers. Ceylan et al. (2007), observed that *H. pylori*-associated infection was (69.2%) and (8%) among mothers in the *H. pylori*-infected and non-infected groups, respectively ($p<0.0001$). Transmission may occur by using common spoons, the licking of teats of feeding bottles, or for chewing or tasting children's food (Rothenbacher et al., 1999).

Although breast feeding has been shown to decrease the risk of infections in infants, especially faeco-orally transmitted infection, no protective effect of breast feeding was reported in this study. Findings of this study agree with other studies that were unable to demonstrate a protective effect of breast-feeding and one study even found that breast feeding increased the risk of childhood infection (Kitagawa et al., 2001; Dore et al., 2002; Rothenbacher et al., 2002). A study performed at USA found that breast-feeding play a protective role against acquisition of *H. pylori* (Malaty et al., 2001). Generally, the relationship between breast-feeding and infection is difficult to be realized as breast-feeding is almost present in the developing world until at least the first year of baby's life.

Contaminated food or water sources have been recognized as important risks for *H. pylori* infection especially in the developing world (Lu et al., 2002). Surprisingly and despite the high prevalence of *H. pylori* infection, all infants included in this study lived in houses with a clean water supply. Lu and colleagues, (2002), findings were in concordance with these findings. These unexpected finding was thought to be due to sub-optimal water treatment or breaks in the municipal pipes allowing for surface contamination of the water. Literature presented some cases, mainly in developing countries, where monitoring showed failures to establish the safety of the water consumed with presence of contaminating microorganisms in drinking water. This was because of limited resources and sanitation standards (Emiliano and André, 2012).

Previous studies on Egyptian children with age ranging from 6 months to school age reported that *H. pylori* prevalence ranged from 15-75.38% (Bassily et al., 1999; Naficy et al., 2000; Mohammad et al., 2007). In most cases this was lower than findings of this study. This discrepancy between results could be due to the difference in studied group ages economic standards or in testing methodology. Also, in Turkey different results were found with respect to the prevalence of *H. pylori* among different pediatric age groups (Us and Hasçelik, 1998; Selimoğlu et al., 2002).

Lower infection rates among Egyptian adults compared to infants can be explained by the results of many studies which found that infection among infants and young children can be transient. In a serology study of Egyptian children between 6 and 36 months of age during the period of observation, 42% of the children had sero-reversion between the first and second blood test, suggesting a spontaneous clearance of the infection (Naficy et al., 2000). Similar findings were reported in Peru, which found an overall prevalence decreased from 71.4% to 47.9% when children were between 6 and 18 months of age (Klein et al., 1994). Thus, while *H. pylori* infections in children appears to have repeated cycles of acquiring and losing the infection until the infection eventually becomes chronic, in adults it is chronic (Goodman et al., 2005).

This decrease in the prevalence with age (Rothenbacher et al., 2002; Broussard et al., 2009), suggested spontaneous eradication, better attention to health issues in older children, or use of antibiotics for other common diseases (Malaty et al., 2002; Rothenbacher et al., 2002). Another explanation of this finding could be an increasing in antibody production with increasing age that may lead to decline of the prevalence rate in older children (Rothenbacher et al., 2002). Other suggested explanations for this decrease in prevalence are differences in types of

H. pylori in adults compared to children and differences in special gastric receptors (Granstrom et al., 1997).

The high prevalence among asymptomatic infants observed in this study (88.9%) parallels that reported in asymptomatic Colombian children (80.2%) (Sicinschi et al., 2012) and an overall prevalence of 86.8% in asymptomatic subjects in South Africa (Dube et al., 2009).

Findings of this study are comparable to findings from other African and Asian countries in which prevalence of *H. pylori* infection ranged from 37.5 to 74.6% (Klein et al., 1994; Kawasaki et al., 1998; Rahman et al., 1998; Thomas et al., 1999; Wizla-Derambure et al., 2001; Hoang et al., 2005; Langat et al., 2006; Hestvik et al., 2010). The reason for the discrepancy between the results is not certain but is likely multi-factorial, including different study methodology, as well as host and environmental factors.

Very few studies have researched the prevalence of virulent *H. pylori* genotypes among Egyptian population and no studies have researched this among asymptomatic Egyptian infants or children. Although, it has been reported that the sensitivity and specificity of *ureA* is more than 90% (Sugimoto et al., 2009), the *ureA* gene was detected in 86.9% of positive cases in this study. This is consistent with results reported by Lu et al. 2002, who found that the sensitivity of *ureA* gene PCR was unsatisfactory and only 75% of specimens were amplified. This low sensitivity may be due to sequence polymorphism.

In the current study, the *cagA* gene was found in 120 of the 168 positive cases (71.4%). No previous trials to study the prevalence of cagA in Egyptian children or infants were done. The prevalence of *cagA* in Egyptian adults, according to earlier studies, ranged from (11.1 - 89%) depending on the clinical presentation of *H. pylori* (van Doorn et al., 1998; Said Essa et al., 2008). The prevalence of the *cagA* gene in children among European countries varies from 22.4 to 76% (Karhukorpi et al., 2000; Oleastro et al., 2003).

The vacA genes were detected in 166/168 (98.8%) of the infected cases, the most predominant type was *s1m2* (50.6 %). The *vacA s1m1, s2m1, and s2m2* genotypes were found in 28.9%, 2.4%, and 18.1%, respectively. It has been demonstrated that the geographic distribution for *vacA* alleles differs in many countries around the world (van Doorn et al., 1998). The *vacA s1m1, s1m2*, and *s2m2* genotypes were found in 34.7%, 57.1%, and 8.2%, respectively with no *s2m1* genotype was detected, in Turkey (Ozbey et al., 2013). The current data is consistent with the results reported in Poland (Maciorkowska et al., 2007) and Shanghai (Zhou et al., 2010) where the *s1m2* was the most prevalent genotype. In contrast, other predominant *vacA* genotypes were reported in Brazil, Slovenia, the Mid-western United States *(s1m1),* and Spain *(s2m2)* (Podzorski et al., 2003; Homan et al., 2009; Agudo et al., 2010; Garcia et al., 2010).

The *babA2* gene was detected in 114/168 (67.8%) of samples. This rate agrees with results obtained from Brazil, and Bulgaria but was higher than that obtained in Portugal and United States (Oleastro et al., 2003; Podzorski et al., 2003; Garcia et al., 2010; Boyanova et al., 2011). The fact that *H. pylori* strains exhibit different patterns of adherence to gastric mucosa cells in adults and children can be an explanation of the low prevalence of *babA2* in children (Blom et al., 2000).

Prevalence of *H. pylori*, as a human pathogen, in the developing world is high. As an objective of this study; prevalence of *H. pylori* virulence genotypes among asymptomatic Egyptian infants (a poorly studied group) was researched. Also, associated risk factors especially those related to human poverty were studied. The high prevalence of infection reported by the current study was alarming. With the high Egyptian birth rate, decline of economic resources after the Egyptian revolution health awareness among many Egyptian mothers, the prevalence of this infection may increase in the future with a possibility of more serious complications. So, more attention towards Egyptian infants' healthcare, improving standards of livings and maternal health education is required.

Conclusion

The prevalence of virulent strains of *H. pylori* among Egyptian infants, based on non-invasive test, was found to be very high in the current study. High prevalence of disease associated genotypes was detected with 41.7% of positive cases were triple positive for *cagA, vacAs1 and babA2*. Bed sharing, premastication of food, bottle feeding, cow milk feeding and nursery attendance were found to increase risk of *H. pylori* infection. Health education and environmental sanitation are recommended to lower prevalence *H. pylori* infection in developing countries. Follow up of those infants is recommended to study the dynamics and complications of infection at this early age.

Conflict of Interests

The authors have not declared any conflict of interests.

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