African Journal of Microbiology Research

Volume 8 Number 5, 29 January, 2014

ISSN 1996-0808

Academic Journals

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

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Vol. 8(5), pp. 437-440, 29 January, 2014
DOI: 10.5897/AJMR2013.6262
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Control of urban soil's toxicity by evaluation of bacterial community changes

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Accepted 20 January, 2014

Since 1990, Almaty City's soils has been considerably polluted by heavy metals, which resulted in its' unhealthy ecological conditions. Almaty soils are light chestnut soils with the following physical and chemical properties: packing, 1.6±0.5 g·cm^{-3}; available air space, 36.1 ± 9.4%; salt composition of the soil aqueous extract, 0.12±0.04%; hygroscopity (water-absorbing quality), 17.2±1.8, quantity of exchangeable cations, mg· eqv·100^{-} g soil- 59.1±4.2 Ca^{2+}, 8.4±0.5 Mg^{2+}, 0.6±0.1 K^{+}, 0.3±0.04 Na^{+}; humus, 1.6±0.4%; C_{total}, 0.9±0.2%; N_{total}, 0.14±0.02. The purpose of this research was to determine the degree of soil toxicity by the method of multisubstrate testing (MST) for the integral studies of Almaty city soils' bacterial communities, polluted by heavy metals (HM). This method measures soil bacterial communities' activity in terms of its uptake intensity of 47 different organic substrates. Their stunted condition was determined through a decreased amount of consumed substrates ("N") and urban soils' metabolic activity decay: total biomass ("W"), load upon ecosystem or disturbance "d" coefficient. It was found that: "d" ecoefficient equaled 1.0 for the baseline soil and "d" ratio equaled 0.6 for urban soils. Ecological soil state was assessed using MST method, looking into soil bacterial communities' activity in terms of its uptake intensity of different organic substrates. This method was developed in the last decade of the twentieth century for the purpose of investigation of soil microbial communities' state. Thus, urban soil samples displayed worse uptake intensity than the baseline soil. Hence, the MST method may be used as the original method of evaluation of urban soil toxicity degree.

Key words: Biological control, multisubstrate testing, bacterial communities, heavy metals, urban soils.

INTRODUCTION

Previously, we attempted to determine the toxicity of soil Almaty by means of phyto-object - Perennial Rye lawn grass (Mynbayeva et al., 2012). In the present study, as a test-object for toxicity, we used the soil microbial communities (bacteria, microscopic fungi, yeast, actino-bacteria, etc.) as indicators of ecosystem degradation processes. But different microflora species and their interpopulation interactions made this task difficult. It is also difficult to obtain reliable results on the state of the soil communities only with the study of structural characteristics. Therefore, it is necessary to define the functional (dynamic) characteristics by other method-logical techniques. The multisubstrate test (MST) is a known method by using the devices of "Ecologist" or "Biologist" (Garland and Mills, 1991; Gorlenko and Kozhevin, 1994; Mills and Bouma, 1997).

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According to the mentioned method, the functional properties of the soil communities are studied by its reaction (direct microscopy) by applying into the soil samples the different artificial substrates, that is, glucose, mannitol, asparagines, citrate, succinate, etc. Further, the method proposed by Yakushev and Byzov (2008), Beleneva et al. (2010), etc. is commonly used in soil and marine microbiology for studying the development of specific active-functioning microbial communities, depending on the applied substrate.

Urban soils' microcenosis initiation by organic substrates till now has not been studied. The purpose of our research was to apply the MST method for the integral studies of Almaty city soils' bacterial communities, polluted by heavy metals (HM), with the use of 47 substrates utilized by these communities.

MATERIALS AND METHODS

To determine the biodiversity and metabolic profiles of urban soils' bacterial communities, soil samples were taken from 5 sectors in 5 replicates of the city along Raiymbek ave. from East to West: No. 1-4- urban soils, No. 5- 25 km from the city (background zonal soil).

The soil samples were collected from each of the 5 sectors during 2005 and 2009 in spring and autumn periods (50 samples) at depth of 0-25 cm (at 5 points) by classical method of soil samples selection. The analysis were performed using an AA-6650 atomic absorption spectrophotometer (Shimadzu Co. Ltd., Japan) equipped with a deuterium lamp which was employed for the determination of heavy metals. All the measurements were based on integrated absorbance. The content of acid-soluble forms of heavy metals was determined after dissolution of soil samples in 1 N HCl, the ratio of soil sample to extra gent was 1:10, exposure time- 60 min.

Multisubstrate testing of microbial communities in Almaty city soils was conducted in a standard way: 2 g of dry-weighed soil was dissolved in the phosphate buffer and centrifuged. Then, 20 ml of supernatant was placed in 200-ml cells of immunological plates for incubation at temperature of 28°C from 12 to 72 h until the coloration of cells may be visually recorded: violet tetrazolium changed to claret color of reduced triphenyltetrazolium. Registration was carried out by using an automated system "Ecologist". The optical density of cell (OD) was measured using a spectrophotometer "Uniplan". Spectrum of consumed substrates (SCS) by the software "Ecologist" was analyzed (Gorlenko and Kozhevin, 1994).

The bacterial communities with a maximum value "d" are considered as optimal and stable; high values "D" are typical for stable systems with high biodiversity and conversely (Garland et al., 1997). For qualitative identification of system type the cluster analysis (Euclid-Ward) was performed. All laboratory analyzes were done in three replications.

RESULTS

In this research, we conducted a comparative study of bacterial communities' metabolic spectrum of certain urban territory areas by MST method. These areas are constantly and chronically polluted with HM, and unpolluted with HM background soil (used as a control). Content of HM required for discussion of obtained results is presented in Table 1.

All analyzed HM were detected in Almaty city soils, but their contents varied depending on the place of soil sampling. The content of Cd and Cu in all urban soils samples exceeded the background: the maximum excess was 4.2 and 2.5 times, respectively. The excess of Pb and Zn in urban soils when compared with the background amounted to 2.6 and 1.5 times, respectively.

Thus, urban soil samples were identified as the most toxic due to high HM content. When using the MST method, we received a multidimensional array of data (range of substrate consumption), which was unique functional "portrait" of studied microbial objects in Almaty soil samples. To determine the patterns of change in the microbial communities' functional profiles the MST data were subjected to cluster analysis. It demonstrated that the urban soils' microbial communities (both spring and autumn samples), sharply differed from the background (control) sample regarding to assimilation of individual substrates, generating separate clusters (Figure 1). This pointed out to the long-term preservation of the effect of HM on the soil microbiota. Seasonal trend prevailed over the regional trend, which was especially expressed for urban soils. The least affected by seasonal trend were the control soil samples of suburban soil that displayed a good level of pedobiota's biodiversity. Characterizing the soil samples by their location in the region, using the extensive signs of functional diversity: the number of consumed substrates "N", metabolic work (a function of total biomass "W", ratio of ecosystem stress or disturbance "d"), showed a significant seasonal dynamics of these parameters.

System stability analysis with respect to consumed substrates by the value of "d" coefficient, pointed to significant differences of background soil (d=1.0) from urban soil (d=0.6), which, in turn, indicated significant changes in the original functional integrity and weak recovery in urban soils. The greatest biodiversity had a background soil sample (Table 2).

It was noted that the microbial communities of background zonal soils in spring fell under stress conditions influenced by melting snow and rapid vegetation, while the urban soils had stress in the autumn, which is probably due to depletion of soil resources at the end of the growing season in view of the overall low productivity of urban ecosystems. Soil bacteria communities were more active in utilization of various organic substrates in the background soil; metabolic diversity and activity fell in the urban soil samples with the maximum HM concentrations.

Conclusion

It was shown that Almaty city's soils were significantly polluted with heavy metals (Pb, Cu, Cd and Zn): metals content exceeded MAC. Heavy metals had a major impact on microbial objectives in Almaty city's soil

Table 1. The content of acid-soluble forms of heavy metals in analyzed soil samples by seasons.

The concentration of acid-soluble forms of heavy metals, mg/kg				
Sector	**Spring samples (average)**			
	Pb	**Cd**	**Cu**	**Zn**
Urban soil	50.3±3.3	0.50±0.04	43.9±2.6	59.8±5.7
Background soil	19.8±1.6	0.16±0.02	21.5±1.9	41.3±4.2
Sector	**Autumn samples (average)**			
	Pb	**Cd**	**Cu**	**Zn**
Urban soil	35.5±5.2	0.17±0.03	28.7±5.2	44.8±8.7
Background soil	15.8±2.1	0.05±0.02	16.3±2.8	28.4±5.2

Table 2. Characteristics of places selection of Almaty city's soil samples obtained by MST method (Garland et al., 1997).

Sector	Parameter "d"	System description
Urban soils	0.9-0.4	A soil system with depleted resources or system that is under the reversible environmental impact of an upsetting factor
Background soil	0.05	A thriving redundant soil system with a maximum margin of safety

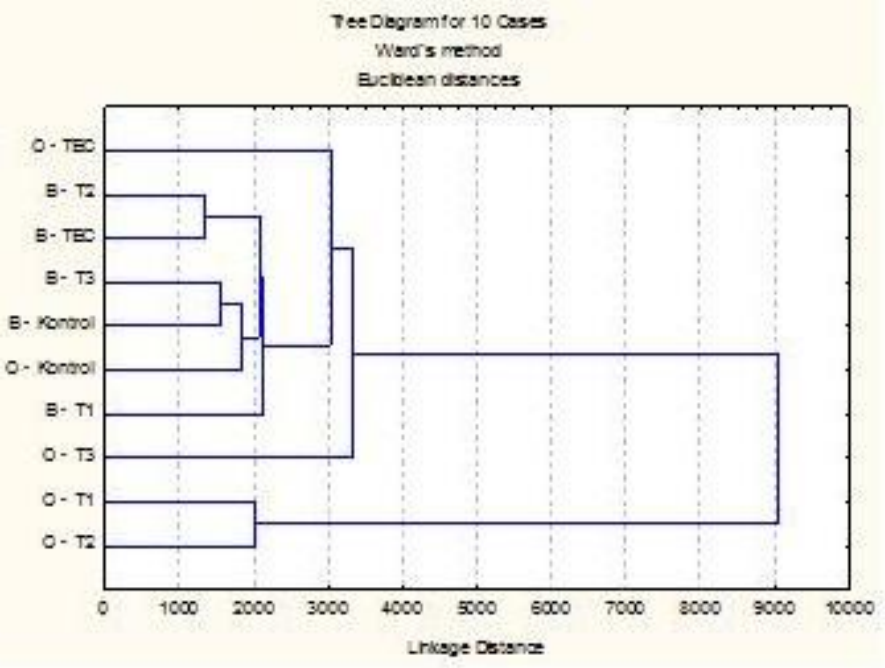

Figure 1. Dendrogram of similarities (by ward) of Almaty soil's microbial communities in urban and background soils (MST method). O- autumn soil samples, B- spring soil samples, urban soils- No.1-4 (T1, T2, T3, TEC), background (No.5)- taken outside the city.

samples.

Urban soil's stunted condition was determined through a decreased amount of consumed substrates N and urban soils' metabolic activity retarded: total biomass W, load upon ecosystem or disturbance d coefficient. For example, disturbance ratio d equaled 1 for the baseline

soil and d ratio equaled 0.6 for urban soils. Coefficient figures received N and W which confirmed low level of functional diversity of soil microbial flora.

The intake of biological substrates was significantly lower in urban soils than in the baseline soil samples, which also gave evidence of the biodegradation of soil in Almaty city. Regularities found have been set both for spring and autumn periods. In springtime stress load (high d) appeared in baseline soil under the influence of snow melting and abrupt vegetation. However, for urban soil stress load appeared in autumn. We connected it with soil depletion at the end of growing season and with overall low urban soil ecosystems productivity. Thus, we established seasonal changes of bacterial biodiversity that prevailed over the spatial parameters. Rural (baseline) soil had good condition characteristics of microbial communities in all the coefficient figures received.

Thus, according to the MST method, the bacterial communities from undisturbed soil oxidized organic substrates are more active than urban soil's communities. These significant differences between soils with different degrees of HM contamination allowed using the MST technique in monitoring of Almaty city soil pollution.

ACKNOWLEDGEMENT

This work was performed under the fundamental researches project of the Ministry of Education and Science of the Republic of Kazakhstan, 2012-2014.

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Vol. 8(5), pp. 406-410, 29 January, 2014
DOI: 10.5897/AJMR10.303
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Human pandemic threat by H5N1 (avian influenza)

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Accepted 26 August, 2013

Influenza viruses infect a wide range of animal hosts and cause yearly wintertime epidemics among people living in temperate zones. Due to their ability to mutate, re-assort gene segments, and cross species, influenza viruses can also lead to pandemics in which immunologically naive people are exposed to a new, highly contagious subtype. In the last century, these pandemics were caused by influenza viruses whose surface attachment proteins, or hemagglutinins, were derived from birds, the natural reservoir of influenza virus. Vaccines are the primary means to provide protection for people at risk for inter-pandemic influenza, and new vaccines, directed against avian-potentially pandemic-strains are now being tested. The aim of this study was to examine available information on influenza pandemic in order to create awareness of preventive measures against influenza pandemic and to suggest future research areas in developing control strategies.

Key words: Influenza, pandemic, H5N1, vaccine, antiviral agents.

INTRODUCTION

Influenza pandemics, defined as global outbreaks of the disease due to viruses with new antigenic subtypes, have exacted high death tolls from human populations. The last two pandemics were caused by hybrid viruses, or reassortants, that harbored a combination of avian and human viral genes. Avian influenza viruses are therefore key contributors to the emergence of human influenza pandemics. Influenza pandemics, defined as global outbreaks of the disease due to viruses with new antigenic subtypes, have exacted high death tolls from human populations (Horimoto and Kawaoka, 2001).

Influenza occurs in both pandemic and interpandemic forms. Fortunately, pandemics, occur infrequently. Interpandemic influenza, although less extensive in its impact, occurs virtually every year (Dolin, 2005). Currently, influenza epidemics in the winter are caused by H3N2 and H1N1 influenza A and influenza B viruses. In addition to seasonal influenza epidemics, influenza pandemic have occurred periodically. An influenza pandemic occurs when an influenza strain with a novel HA subtype (with or without a novel NA subtype) appears and spreads in the human population, which has little or no immunity to the novel HA. In the 20th century, pan-demics occurred in 1918, 1957, and 1968 and were associated with substantial illness and death (Luke and Subbarao, 2006). Influenza A viruses are perpetuated in the wild birds of the world, predominantly in waterfowl, in which the 16 subtypes coexist in perfect harmony with their hosts (Webster et al., 2006).

During March 2006-March 2009, a total of 6,355 suspected cases of avian influenza (H5N1) were reported to the Ministry of Health in Egypt. Sixty-three (1%) patients had confirmed infections; 24 (38%) died. Risk factors for death included female sex, age > or = 15 years, and receiving the first dose of oseltamivir >2 days after illness onset. All but 2 case-patients reported exposure to domestic poultry probably infected with avian influenza virus (H5N1). No cases of human-to-human transmission were found. Greatest risks for infection and death were reported among women > or = 15 years of age, who

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accounted for 38% of infections and 83% of deaths. The lower case-fatality rate in Egypt could be caused by a less virulent virus clade. However, the lower mortality rate seems to be caused by the large number of infected children who were identified early, received prompt treatment, and had less severe clinical disease (Kandeel et al., 2010). In Egypt, influenza types and subtypes are: H1N1, H1N2, H3N2 and B. One or two types are usually circulating in a season. In the last 2 years, influenza in Egypt has taken on special importance and attracted media attention since that season, also because the H5N1 strain attacked poultry in 2006 and was detected in some Egyptian human cases. In 2005 and 2006, both types A and B were circulating. The subtype A/H1N1 was circulating in 2006 (Awadalla et al., 2009).

PANDEMIC PHASES

In reviewing the public health implications of a pandemic, it is useful to understand the various phases that a pandemic will go through. World Health Organisation (WHO) has developed these phases that can be used by pandemic planers (WHO Influenza pandemic preparedness, 2005).

Interpandemic period

Phase 1

No new influenza virus subtypes have been detected in humans. As influenza virus subtype that has caused human infection may be present in animals. If present in animals, the risk of human infection or disease is considered to be low.

Phase 2

No new influenza virus subtypes have been detected in humans. However, a circulating animal influenza virus subtype poses a substantial risk of human disease.

Pandemic alert period

Phase 3

Human infection(s) with a new subtype, but no human-to-human spread, or at most rare instances of spread to a close contact.

Phase 4

Small cluster(s) with limited human-to-human transmission but spread is highly localized, suggesting that the virus is not well adapted to humans.

Phase 5

Larger cluster(s) but human-to-human spread still localized, suggesting that the virus is becoming increasingly better adapted to humans, but may not yet be fully transmissible (substantial pandemic risk).

Pandemic period

Phase 6

Pandemic refers to increased and sustained transmission in general population.

Evolutionary pathways of influenza viruses

Studies on the ecology of influenza viruses have led to the hypothesis that all mammalian influenza viruses are derived from avian influenza reservoirs support for this theory comes from phylogenetic analyses of nucleic acid sequences of influenza A viruses from a variety of hosts, geographic regions, and virus subtypes (Webster et al., 1992).

A surprising discovery from phylogenetic analyses of amino acid changes was that avian influenza viruses, unlike mammalian strains, show low evolutionary rates (Gorman et al., 1990). In fact, influenza viruses in wild aquatic birds appear to be in evolutionary stasis, with no evidence of net evolution over the past 60 years. Nucleotide changes have continued to occur at a similar rate in avian and mammalian influenza viruses, but these changes no longer result in amino acid changes in the avian viruses, whereas all eight mammalian influenza gene segments continue to accumulate changes in amino acids. The high level of genetic conservation suggests that avian influenza viruses in their natural reservoirs are approaching or have reached an adaptive optimum, wherein nucleotide changes provide no selective advantage. It also means that the source of genes for pandemic influenza viruses exists phenotypically unchanged in aquatic bird reservoir (Wright and Webster, 2001).

This lack of change is surprising because influenza viruses are segmented, negative-stranded RNA viruses that have no quality control mechanisms during replication and are highly prone to variation. However, all 16 HA subtypes, including H5N1, have until recently been considered to be benign in their natural hosts. This benign equilibrium between the influenza virus and its host may have changed (Webster et al., 2006).

Overall, the most important implication of phylogenetic studies is that the ancestral viruses that caused Spanish

influenza in 1918, as well as the viruses that provided gene segments for the Asian/1957 (H2N2) and Hong Kong /1968 (H3N2) pandemics , are still circulating in wild birds, with few or no mutational changes (Wright and Webster, 2001). The startling observation of Taubenberger and Morens (2006) was that the 1918 virus did not originate through a reassortment event involving a human influenza virus: all eight genes of theH1N1 virus are more closely related to avian influenza viruses than to influenza from any other species, indicating that an avian virus must have infected humans and adapted to them in order to spread from person to person. Thus, pandemic influenza may originate through at least two mechanisms: reassortment between an animal influenza virus and a human influenza virus that yields a new virus, and direct spread and adaptation of a virus from animals to humans.

It is important to mention influenza A/ H1N2 viruses, which emerged during 2001, are genetic reassortants between H1N1 and H3N2 subtype viruses which have cocirculated in the human population since 1977. They possess a H1 hemagglutinin antigenically and genetically similar to contemporary A/New Caledonia/20/99 (H1N1)-like viruses and seven genes closely related to those of recent A/Moscow/10/99 (H3N2)-like viruses. The viruses have spread to many regions of the world and have predominated over H1N1 viruses in several countries (for example, Egypt) (Gregory et al., 2002).

Next pandemic

Past influenza pandemics occurring during the 20th century apparently all arose from the Eurasian avian lineage of viruses. Over the past several years, a great deal of attention has been focused on the role of avian influenza viruses as the source of the next pandemic strain (Horimoto and Kawaoka, 2001). Avian influenza was first identified in Italy more than 100 years ago. Pigs have receptors for avian and human influenza viruses and are susceptible to both; therefore, pigs have been considered logical intermediary hosts for viral reassertment between avian and human influenza strains (Horimoto and Kawaoka, 2001). However, the role of pigs in creation of pandemic strains is still not clear. It is also not clear if reassortment in another animal host is necessary or whether an avian strain could directly cause a global pandemic in humans (Webster, 1997).

The last two pandemic viruses were combinations of bird and human influenza viruses (wild birds are considered the reservoir for type A influenza viruses). Many persons believe that these new viruses emerged when an intermediate host, such as pig, was infected by both human and bird influenza A viruses at the same time. A new virus was created. Events in Hong Kong in 1997, however, showed that this is not the only way that human can become infected with a novel virus. Some-times, an avian influenza virus can jump the species barrier and move directly from chickens to humans and cause the disease. So the direct contact with infected poultry is the route of transmission (Chotpitayasunondh et al., 2006). In addition to a growing list of avian species that can be infected with H5N1 virus, the virus has infected several mammalian species, including tigers, leopards and pigs (Fauci, 2006). Influenza viruses are impossible to eradicate, as there is a large reservoir of all subtypes of influenza A viruses in wild aquatic birds. In agricultural - based communities with high human population density such as are found in China, conditions exist for the emergence and spread of pandemic viruses. It is also impossible to predict when the next pandemic will occur. Moreover, the severity of illness is also unpredictable, so contingency plans must be put in place now during the inter-pandemic period. These plans must be flexible enough to respond to different levels of disease (Cox et al., 2003).

Influenza A (H1N1) virus emerged in 2009. It is a new reassortment that has never before circulated among humans. This virus is not closely related to previous or current human seasonal influenza viruses. Respiratory transmission occurs mainly by droplets disseminated by unprotected coughs and sneezes. Short-distance airborne transmission of influenza viruses may occur, particularly in crowded enclosed spaces. Hand contamination and direct inoculation of virus is another possible source of transmission (WHO, 2013b).

Influenza A (H7N9) is one of a subgroup of influenza viruses that normally circulate among birds. Until recently, this virus had not been seen in people. However, human infections have now been detected. As yet, there is limited information about the scope of the disease the virus causes and about the source of exposure. The disease is of concern because most patients have been severely ill. There is no indication thus far that it can be transmitted between people, but both animal-to-human and human-to-human routes of transmission are being actively investigated (WHO, 2013a).

We cannot predict when the next influenza pandemic will occur, or which influenza virus subtype will cause it. Forecasts of the severity of the next influenza pandemic differ in their predictions of deaths based on the models used. Modeling based on the pandemic of 1968 projects 2 million - 7.4 million excess deaths worldwide (Luke and Subbarao, 2006).

The H5N1 virus has infected birds in more than 30 countries in Asia, Europe and Africa, while further geographical spread remains likely. Human infections are still rare and the virus does not spread easily from birds to humans or readily from person to person (Saeed and Hussein, 2006) The epidemic of H5N1 highly pathogenic avian influenza in Southeast Asia raises serious concerns that genetic reassortment will result in the next influenza pandemic. There have been 164 confirmed cases of

human infection with avian influenza since 1996. In 2004 alone, there were 45 cases of human H5N1 in Vietnam and Thailand, with a mortality rate over 70%. In addition to the potential public health hazard, the current zoonotic epidemic has caused severe economic losses (Zeitlin and Maslow, 2006). Since 2003, there have been a total of 436 cases and 262 deaths due to H5N1 infections. The number of cases has decreased steadily since 2006 (Adams and Sandrock, 2010).

In six countries this virus has also caused fatal human infections. This has sparked fears that this agent may be the progenitor of a new pandemic influenza virus. During summer 2005 the disease has slowly spread westward. Isolated outbreaks have been reported from Kazakhstan, Russia, Romania, Turkey, Croatia and Ukraine. Migratory birds have been tentatively accused for spreading the infection along their flyways (Werner, 2006).

This rapid rate of spread of virus along with notoriety of the virus for frequent genetic re-assortment, which might enable H5N1 to infect human beings, threatens of possible influenza pandemic since the last pandemic in 1968. The human influenza caused by this subtype of the virus (H5N1) has high case fatality of 54% and majority of affected humans are between the ages of 5 to 23 years (Lahariya et al., 2006).

Human infection with avian influenza virus

Influenza A viruses causes natural infections of humans, some other mammals and birds. Few of the 16 haemagglutinin and nine neuraminidase subtype combinations have been isolated from mammals, but all subtypes have been isolated from birds (Alexander, 2006), of the 16 avian influenza virus subtypes, H5N1 is of particular concern for several reasons:

1. H5N1 mutates rapidly and has a documented propensity to acquire genes from viruses infecting other animal species. Its ability to cause severe disease in humans has now been documented (WHO: Avian influenza, fact sheet, 2004).
2. The virus has spread rapidly throughout poultry flocks in Asia over the past 2 years and now appears to be endemic in eastern Asia (Kaye and Pringle, 2005).
3. It has shown a propensity to acquire genes from viruses infecting other animal species. It causes severe disease in humans, with a high case-fatality rate (reportedly at about 70%, although adequate surveillance data are lacking to accurately define the rate).
4. The potential of exposure and infection of humans is likely to be ongoing in rural Asia, where many households keep free-ranging poultry flocks for income (Stohr, 2005).

The emergence of multiple genetically distinct sublineages of H5N1 has continued. These emerging sublineages display varying levels of drug resistance and in some cases an increased preference for binding to human α2,6-linked sialic acid cellular receptors. Though H5N1 has not shown efficient transmissibility between humans, the rapid evolution of the virus presents a concern for the emergence of a virus with this capability (Adams and Sandrock, 2010). Vaccination is the best option by which spread of a pandemic virus could be prevented and severity of disease reduced. Production of live attenuated and inactivated vaccine seed viruses against avian influenza viruses, which have the potential to cause pandemics, and their testing in preclinical studies and clinical trials will establish the principles and ensure manufacturing experience that will be critical in the event of the emergence of such a virus into the human population (Luke and Subbarao, 2006).

Inactivated vaccines against avian influenza subtypes require two doses and administration with adjuvant to achieve the desired level of the neutralizing antibody. The precise antigenic properties of a nascent pandemic strain cannot be predicted, so available vaccines may be poorly antigenically matched to the pandemic virus. Manufacturing capacity, the ability of candidate vaccine stains to grow well in eggs, and biological safety containment of parent strains for vaccine development are all problems to be addressed. Efforts are under way to develop and evaluate live, attenuated vaccines against potential pandemic strains of influenza along a track that parallels the development and evaluation of inactivated virus vaccines (Luke and Subbarao, 2006). To date, vaccines have been shown to be safe and well tolerated, but have required multiple doses and dosage levels higher than traditionally needed for seasonal influenza vaccines in order to generate immune responses thought to be protective (Campbell, 2006). If the emerging avian influenza or another new virus creates a pandemic, severely limited supplies of vaccines and antiviral medications are likely (Temte, 2006). Efforts must be concentrated on early detection of bird outbreaks with aggressive culling, quarantines, and disinfection. To prepare for and prevent increased human cases, it is essential to improve detection methods and stockpile effective antiviral (Zeitlin and Maslow, 2006).

Since 2005 it is recommended that people with occupational contact with wild or domestic birds should be vaccinated to reduce the risk of simultaneous infection with a human and an avian influenza virus (Eich, 2007). Development of effective vaccines against highly pathogenic avian influenza H5N1 viruses with the potential to cause a pandemic is a public health priority (Hoelscher et al., 2008). A two-dose vaccine regimen of either 7.5 or 15 mg of hemagglutinin antigen without adjuvant induced neutralizing antibodies against diverse H5N1 virus strains in a high percentage of subjects, suggesting that this may be a useful H5N1 vaccine (Ehrlich et al., 2008). Another randomised, dose comparison, parallel assignment, multicentre trials conducted in Australia, healthy adult volunteers received two doses

of vaccine (phase I trial; N=400, phase II trial; N=400) (Nolan et al., 2008).

Antiviral agents can be used to treat influenza infection and can be taken as chemoprophylaxis during influenza outbreaks (Stephenson and Democratis, 2006). Oseltamivir (Tamiflu®) has been shown to be effective in the treatment and prevention of epidemic influenza infection in adults, adolescents and children (≥ 1 year).

Although oseltamivir has not been approved for prophylactic use in children, it has been shown to be effective. Oseltamivir is also active against avian influenza virus strains. Evidence suggests that lower doses or shorter durations of treatment/chemoprophylaxis other than those approved may not be effective and may contribute to emergence of viral resistance (Ward, et al., 2005).

CONCLUSION

Avian influenza refers to a large group of different influenza viruses that primarily affect birds. On rare occasions, these bird viruses can infect other species, including pigs and humans. The vast majority of avian influenza viruses do not infect humans. An influenza pandemic happens when a new subtype emerges that has not previously circulated in humans. An influenza pandemic is a rare but recurrent event. Three pandemics occurred in the previous century: “Spanish influenza” in 1918, “Asian influenza” in 1957, and “Hong Kong influenza” in 1968. The keystone of influenza prevention is vaccination; vaccine preparation should be by genetic reassortment of high-yield seed viruses of all hemagglutinin subtypes. The recommendation assumes that the next pandemic virus is unpredictable, but it will come from one of the 16 hemagglutinin subtypes of avian or mammalian strains of influenza A.

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Vol. 8(5), pp. 411-416, 29 January, 2014
DOI: 10.5897/AJMR2013.6540
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Review

Newcastle Disease: Present status and future challenges for developing countries

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Accepted 13 January, 2014

Newcastle disease (ND) is an important infectious disease of the poultry that is caused by virulent strains of Avian Paramyxovirus - 1, which is a single strand non segmented negative sense RNA virus. The virus belongs to family Paramyxoviridae and it has 10 serotypes designated as APMV-1 to APMV-10. The Newcastle disease virus (NDV) is endemic in many countries of the world. The first outbreak of Newcastle disease was observed at Newcastle during 1926. Later, it was found in various parts of the world. NDV spreads mostly by the direct contact between healthy and diseased birds or also by the excretions of infected birds. This disease can vary in nature from mild to severe depending upon the type of the virus. NDV can infect both domestic and wild birds. This disease can have a devastating effect on poultry due to high morbidity and mortality rates. In unvaccinated chickens, the morbidity and mortality rates may reach up to 100% each, depending upon the virulence of the NDV. Live virus vaccines and killed oil based vaccines are used in many countries to prevent the disease in chickens. Despite the extensive use of vaccines, outbreaks are continuously occurring in various parts of the world resulting in huge losses since few years. Moreover, extensive use of vaccines has made the situation favorable for genetic modification of the pathogenic virus. Keeping these issues in mind, future challenges are highlighted in detail.

Key words: Newcastle disease, vaccines, outbreaks, genetic modification, avian paramyxovirus-1.

INTRODUCTION

Newcastle disease (ND) is one of the most important viral diseases (Orsi et al., 2010). It is an acute infectious viral disease of domestic poultry and other species of birds regardless of variation in sex and age (Alexander, 2003; Haque, 2010; Iram et al., 2013). ND causes huge economic losses to the commercial poultry farmers round the world (Aldous et al., 2003; Qin et al., 2008; Diel et al., 2012). Etiological agents of ND are virulent strains of avian paramyxovirus - 1 (Qin et al., 2008; Yu et al., 2001; Choi et al., 2010). The disease is characterized by respiratory, nervous system impairment, gastrointestinal and reproductive problems (Nanthakumar et al., 2000; Tiwari et al., 2004). Newcastle disease is commonly known as Ranikhait disease in India (Narayanan et al., 2010; Ravindra et al., 2009) and also in Pakistan.

Newcastle disease virus (NDV) has a wide host range, including approximately 241 species of 27 orders, out of known 50 orders of birds (Madadger et al., 2013). More commonly affected species include chickens, turkeys, ducks, pigeons, (Zhang et al., 2011) guinea fowl, Japanese quail and many wild birds of all ages (Nanthakumar et al., 2000). The most susceptible avian species to this disease are chickens (Rezaeianzadeh et al., 2011) and also some mammals like humans, cats and dogs. During the last 40 years, paramyxoviruses were isolated from different animals (Miller et al., 2009).

In several developing countries, ND is endemic and has greatest impact on villages where people's livelihood depends upon poultry farming (Mohamed et al., 2011; Rezaeianzadeh et al., 2011). APMV-1 viruses circulating

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in poultry flocks are being characterized (Munir et al., 2012). ND is fatal and still top ranked poultry disease. Annual losses caused by this disease worldwide are in millions of dollars (Waheed et al., 2013; Susta et al., 2010).

ND is an economically important disease and also a major threat to poultry industry (Narayanan et al., 2010). According to variation in strains of NDV, the rate of mortality and morbidity in a flock (Haque et al., 2010) varies from 90-100% (Nanthakumar et al., 2000) along with decrease in egg production (Choi et al., 2010).

Due to the severe nature of Newcastle Disease and the related consequences, NDV is included in "LISTED" agents (reportable disease) by Office International des Epizooties (OIE) (Aldous and Alexander, 2001; Boynukara et al., 2013). Notification is required by OIE of any outbreak of ND (Cao et al., 2013), when it meets certain criteria of virulence (Cattoli et al., 2011; Munir et al., 2012).

EPIDEMIOLOGY

The epizootics of Newcastle Disease in poultry continue to occur in Asia, Africa, Central and South America while in Europe, sporadic epizootics occur (Naveen et al., 2013). ND is reported consistently from all continents of the globe (Munir et al., 2012).

Major panzootics of ND have been recorded from different parts of the world. The very first panzootic started in 1926 in Southeast Asia from Java, Indonesia and in Europe from Newcastle-upon-Tyne, England (Seal et al., 1995; Arifin et al., 2011), and it remained till late 1950s (Qiu et al., 2011). The second panzootic began in Middle East in late 1960s and spread to other countries till 1973.

The third drastic panzootic caused by neurotropic form of NDV, termed pigeon paramyxovirus type 1 virus, appeared in Middle East about in the late 1970s. In 1981, ND reached Europe then spread rapidly throughout the globe (Mase et al., 2002). The latest and fourth pandemic emerged by late 1980s in Far East, South Africa, and Europe (Qiu et al., 2011). A sporadic form of Newcastle Disease exists in Pakistan throughout the year; only a limited number of outbreaks are reported annually (Munir et al., 2012a). In Southeast Asia, it is endemic and a cause of huge economic losses to commercial poultry (Munir et al., 2012 b).

During 2012, severe outbreak of ND occurred in Jallo Wildlife Park in Lahore, Pakistan, caused by APMV 1 serotype. Within a week, it took the lives of approximately 190 peacocks with a 100% mortality rate and 50% loss of the susceptible birds. Isolation of virus and serological diagnostics, such as HI Test, ELISA and molecular diagnostic tests like real time PCR confirmed the presence of velogenic Newcastle Disease Virus (Munir et al., 2012c).

ECONOMIC IMPACT

Proteins are a significant part of balanced human diet. There are mainly two proteins sources which are Animals and Plants. In developing countries, human diet is deficient in the animal proteins; approximately 66% population has protein deficient diet (Maqbool, 2002). A single person per day requires 102.7 g protein, while only 69.61 g protein is used by a person per day. The main animal protein sources are mutton, beef, poultry meat, eggs, and milk (Maqbool and Bakhsh, 2007). White meat's essential nutrients are same as red meat, but white meat has the advantage of containing less cholesterol and saturated fat. In most developing countries, meat is a very important protein sources in diet of people because it is affordability and has high quality protein (Thomazelli et al., 2012). In developing countries, the broiler meat is the cheapest source of animal protein. Availability of egg is increasing at rate of round about 4% annually (Numan et al., 2005).

Poultry production was started as a cottage industry in many developing countries of the world. The production and management for disease control measures were not sufficient because of the lack of scientific knowledge. In Pakistan, approximately 1105.91 million poultry birds are present, from which rural poultry is about 152.44 millions. In village economy, it plays vital role with the contribution of about 3611 million eggs and 100.42 metric tons of the total poultry meat (Khan et al., 2010).

Recent studies by Pakistan Economic Survey (2011-2012) reported that poultry sector generates income and direct and indirect employment for about 1.5 million people till 2012. Its contribution in agriculture is 6.40% and in livestock 11.50%. In total, meat production of country and poultry meat contributes 25.8%. Poultry sector has rapid growth of about 8-10% every year, which shows its inherent potential. According to currently conducted survey, the present investment in the Pakistan poultry industry is about Rs. 200.00 billion.

ND and avian influenza (AI) are major concerns of animal husbandry due to hazardous infections (Ge et al., 2012). All over the world, poultry industry is facing severe economic losses with every passing year (Haque et al., 2010; Khan et al., 2011).

ETIOLOGY

According to taxonomy of virus, NDV belongs to order Mononegavirales, family Paramyxoviridae and subfamily Paramyxovirinae (Cattoli et al., 2011). The subfamily is divided into five genera: Mor-billivirus, Respirovirus, Henipavirus, Rubulavirus, and Avulavirus (Miller et al., 2009); all the avian paramyxoviruses APMVs are part of genus Avulavirus. The virus exists in 10 serotypes; APMV-1 to APMV-10 (Waheed et al., 2013), but all NDV isolates belong to serotype 1 (APMV-1). APMV-1 is

synonymous with NDV (Cattoli et al., 2011; Miller et al., 2009). Virions are roughly spherical; 150 nm or more in diameter and filamentous (Catroxo et al., 2011). The genome is about 15.2 kb in length (Cao et al., 2013; Zhang et al., 2012) that codes for six structural and two non-structural proteins (Choi et al., 2010). 'Rule of six' should be followed by genome because it should be of polyhexameric length to replicate rapidly. It encodes for six proteins in 3' to 5' direction; these are Nucleoprotein (NP), Large RNA polymerase (L), Fusion (F), Hemagglutinin Neuraminidase (HN), Matrix (M) and phosphorprotein (P) (Linde et al., 2011; Al-habeeb et al., 2013). The proteins W and V are additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Linde et al., 2011; Qiu et al., 2011).

In virus particles, NP is the most abundant protein which provides the NDVs core helical nucleocapsid structure. NP is the main regulator in replication of viral genome (Kho et al., 2004). The genomic RNA is associated with NP, P and L proteins to form RNP complex, which serve as template for RNA synthesis (Kho et al., 2003). NP is found to be highly immunogenic, as it induces antibody responses in chickens (Ahmad-Raus et al., 2009).

During a field study in Pakistan, 5% of the field isolates were reported as velogenic, 55% as mesogenic and 40% as lentogenic (Waheed et al., 2013). For chickens, different strains of NDV have great variation in pathogenicity. On the basis of clinical signs in infected chickens, strains of NDV are grouped in to five pathotypes: 1) Asymptomatic enteric: a form that has subclinical enteric infection without clear symptoms; 2) Lentogenic: virus present with the mild respiratory infections; 3) Mesogenic: virus presents with rare nervous and respiratory signs while mortality rate is related with the age of susceptible birds (young birds are more susceptible as compare to adults); 4) Viscerotropic velogenic: virus cause haemorrhagic intestinal lesions it is highly pathogenic; 5) Neurotropic velogenic: virus cause high mortalities followed by respiratory and nervous signs (OIE, 2012).

The NDV isolates are differentiated on the basis of *in-vivo* estimation of pathogenicity (Pham et al., 2005). These *in-vivo* tests are mean death time (MDT) in SPF embryonated eggs of chicken, Intracerebral pathogenicity index (ICPI) in 1 day old SPF chicks, and Intravenous pathogenicity index (IVPI) in six weeks old SPF chicks (Wise et al., 2004; Adi et al., 2009; Mohamed et al., 2011). The MDT classifies ND virus strains into the groups: velogenic (takes less than 60 h to kill); mesogenic (takes from 60 to 90 hto kill); and lentogenic (takes more than 90 h to kill). The ICPI classifies ND virus strains by giving indices scores from 2.0 to 0.0. The maximum score of 2.0 is given to most virulent ND virus strain while lentogenic strains are given score close to 0.0. The IVPI classifies the ND virus strains from lentogenic to velogenic. Lentogenic strains and some mesogenic strains have IVPI values of 0.0, whereas the maximum IVPI indices for a virulent strain is 3.0 (OIE, 2004).

MOLECULAR BASIS OF PATHOGENICITY

The genome of NDV encodes for six major structural proteins. Viral replication, transcription and translation occur in the cytoplasm of the host cell, while virus particles are assembled in plasma membrane by budding (Zanetti et al., 2003). Important pathogenic marker of NDV exists in F protein (Madadgar et al., 2013). Disulphide linkage is present between F_1 and F_2. These proteins enable the virus to attach to the host cell membrane (Wen et al., 2007). At cleavage site, F_0 protein has two pair of basic amino acids that can be cleaved by the host proteases (Pham et al., 2005). Highly virulent NDV has three or more basic amino acids, which are lysine (K) or arginine (R) present at 113 - 116 residues and phenylalanine (F) at position 117 (OIE, 2012). Cleavage of F_0 protein is due to the presence of these basic amino acids in virulent NDV (Boostani et al., 2013). It has been found that avirulent viruses have ^{112}G/E-K/R-Q-G/E-R-L^{117} and virulent viruses have ^{112}R/K-R-Q-K/R-R-F^{117} amino acid sequence at cleavage site (Pham et al., 2005). Most of the pathogenic APMV-1 viruses for chicken have sequence ^{112}R/K-R-Q/K/R-K/R-R^{116} (Choi et al., 2010). Office of International Epizootics (OIE) accepts F cleavage sequence as determinant of primary virulence (Wise et al., 2004). However, if this cleavage sequence is not found, then an Intra Cerebral Pathogenicity Index (ICPI) is required for determination of the virulence.

TRANSMISSION

NDV can infect more than 240 species of birds and it spreads primarily through direct contact between healthy and infected birds. The disease transmits through droppings and secretions from the nose, mouth and eyes of infected birds. The disease spreads by contaminated water, feed and transport. Airborne transmission of the virus is also an important route of transmission for ND (Li et al., 2009).

Mechanical transfer of infected faeces occurs by rodents, insects, dogs, fleas, or scavenging animals (Ullah et al., 2004). Infection takes place by virus inhalation, ingestion or by contact with conjunctiva. The disease may vary from subclinical with no mortality to severe infection, with 100% mortality.

SIGNS AND SYMPTOMS

Clinical signs are dependent on factors such as the virus strain, host species, age of the host, co-infection with

other micro-organisms, environmental stress, and immune status (Al-Habeeb *et al.,* 2013). In chickens, the general symptoms are loss of appetite, listlessness, abnormal thirst, weakness, drop in egg production, air sacculitis, tracheitis and conjunctivitis. Respiratory signs can include sneezing, gasping for air, nasal discharge and coughing, whereas a clear intestinal symptom is a greenish watery diarrhea. Nervous symptoms may consist of paralysis of wings and/or legs, twisting of head and neck or complete paralysis (Bhaiyat et al., 1994). Layers show drop in egg production and misshapen soft egg shells (Hadipour et al., 2011). In acute and severe cases (like neurotropic velogenic strain), death is very sudden and birds die without showing any clinical signs. Dead birds have hemorrhagic or necrotic lesions in mucosa of intestine, cecal tonsils, proventriculus and gizzard. Swollen kidneys and deposition of urates are also common lesions.

DIAGNOSIS

Rapid and accurate diagnosis of ND outbreak is important because it clinically resembles highly pathogenic avian influenza (AI) (Khan et al., 2010). Clinical diagnosis based on history, signs and lesions may establish a strong index of suspicion but the laboratory confirmation must be done. Hemagglutination and hemagglutination inhibition test, virus neutralization test, Enzyme linked immune-sorbent assay, plaque neutralization test and reverse-transcriptase polymerase chain reaction (RT-PCR) can be used for confirmation of the ND virus (Chaka et al., 2013). Now RT-PCR is the most exclusively used method to detect AIVs and NDVs (Liu et al., 2011; Haque et al., 2010; Wakamatsu et al., 2007). RT-PCR assay is more sensitive, specific and less labor intensives as compare to other conventional methods used for lab diagnoses such as virus isolation, Immuno-Fluorescence Staining, Neuraminidase Inhibition and ELIZA (Tang et al., 2012; Shahzad et al., 2011). Using modern technologies, new diagnostic techniques are being developed for identification and differentiation of NDV strains (Rezaeianzadeh et al., 2011). Other molecular diagnostic tests like real time PCR and nucleotide sequence analysis are also important in viral disease diagnosis (Shabbir et al., 2012; Shah et al., 2011).

PREVENTION AND CONTROL

Vaccines are being used to control and prevent ND. Currently, many inactivated and live ND vaccines are available around the world (Shim et al., 2011; Xiao et al., 2013). Chickens and turkeys are immunized against Newcastle disease. Live virus vaccines are administered by variety of routes and schedules from hatching till grow-out (Cho et al., 2008). Killed virus oil emulsion vaccines are administered parentally prior to the onset of egg production. Although proper vaccination protects the birds from clinical disease but it does not prevent virus replication and shedding, which results in a source of infection (Chukwudi et al., 2012).

Therefore, the prophylactic vaccination is not used in developed countries (OIE, 2012). In developing countries, there is wide use of vaccines on commercial flocks (Munir et al., 2012b). Anti NDV antibody titers of flocks are continuously monitored and flocks are revaccinated to maintain the protective antibody titers. The breeders and layers are vaccinated against NDV and oil based vaccines are being used prior to onset of egg production for long term immunity (Nadeem et al., 2004). Anti NDV antibody titers of breeder flock is also important to maintain the anti NDV maternal antibody titers of progeny. These maternal antibodies protect chicks from the disease during the first week of life. In spite of extensive vaccination, outbreaks are continuously occurring (Shabbir et al., 2012). To overcome this problem poultry producers are using different com-binations of live and killed vaccines in a flock.

Good biosecurity measures are essential to prevent Newcastle disease in poultry flocks. Commercial flocks should not have any contact with domesticated poultry or wild birds or any pet birds. Workers should avoid contact with birds outside the farm. Biosecurity measures include bird-proof houses, feed and water supplies, minimizing travel on and off the facility, disinfecting vehicles and equipments that enter the farm. Pests such as insects and mice should also be controlled. If possible, employees should shower and change into dedicated clothing prior entry into the poultry farm.

PUBLIC HEALTH

Humans are among the many species that can be infected by NDV in addition to avian species. NDV may cause conjunctivitis in humans, when a person has been exposed to large quantities of the virus (Alexander, 2000). Mostly, Laboratory workers and vaccinators are affected.

The use of personnel protective equipment and biological safety cabinet has reduced the exposure of laboratory workers. Infection is rarely seen in the workers of a farm; moreover persons handling or consuming poultry products do not appear to be at risk (Nolen, 2003).

The conjunctivitis usually resolves rapidly, but the virus will be shed in the ocular discharges from 4 to 7 days. In some cases, mild, self limiting influenza like disease with fever and headache has also been reported in humans (Alexander, 2000; OIE, 2012). There is no evidence found to support human to human transmission but the potential for human to bird transmission exists (Alexander, 2000; David and Daniel, 2003).

FUTURE CHALLENGES

The Newcastle disease virus has not been studied for its evolutionary origin among various outbreaks time to time. Most of the research work was focused on immunological properties of the virus rather than the genomic properties. Further, the extensive use of vaccines makes the situation more favorable for genetic modifications in pathogenic strains. Therefore in International interest, it is essential to address these issues by conducting research on the following lines: 1) Isolation and molecular characterization of velogenic strains of NDV; 2) complete genome sequence analysis of different NDV isolates for further studies of epidemiology, vaccinology and evolutionary origin; 3) existing real time PCR assays should be validated and measures should be devised for prevention and control of epidemics in future.

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Vol. 8(5), pp. 417-424, 29 January, 2014
DOI: 10.5897/AJMR2013.6137
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Quantitative suspension tests for the evaluation of bactericidal, fungicidal and sporicidal effects of biocides used in vaccine production facility

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Accepted 8 January, 2014

The appropriate use of biocides is essential in any vaccine production facility and their proper evaluation using standardized tests marks the first step to ensure their proper use. Quantitative suspension tests against reference and environmental isolates were carried out to evaluate the efficacy of various biocides used in the main vaccine production facility in Egypt. Several use-dilutions of the biocides were evaluated at contact times of up to 5 min in case of antiseptics and 30 min in case of disinfectants to measure the biocide activity against bacteria, fungi and spores. Standard strains were used in addition to the main bacterial isolates identified in an environmental monitoring program carried out in the same facility. Alcohol based biocides showed bactericidal and fungicidal activity but no sporicidal activity. Chlorine based compounds and glutaraldehyde showed bactericidal and fungicidal effect while, the sporicidal effects depended on the used dilution and the contact time. Hydrogen peroxide showed bactericidal, fungicidal and sporicidal activity. Quaternary ammonium compounds tested showed very weak activity in all tests. Evaluation of the biocide is an essential step to guarantee the use of the most appropriate agent in any location.

Key words: Biocides, antiseptics, disinfectants, vaccines, Egypt.

INTRODUCTION

The proper use of biocides is an essential step in decontaminating personnel and environment in any health care settings like vaccine production facilities (Rutala et al., 1998; Kampf et al., 2003; Pineau et al., 2008). Biocides are classified as either antiseptics or disinfectants. Antiseptics are chemical agents that inhibit or kill microbial growth and are nontoxic when applied to living tissues; they are mainly used for hand washing or for treating mucous membranes and surface wounds (Kramer, 2000). Disinfectants are chemical or physical agents used to destroy or irreversibly inactivate many or all of the pathogenic microorganisms but not necessarily spores and not all viruses on inanimate objects (Madigan et al., 2002). Under certain circumstances, the same compound can be used as antiseptic in low concentration and disinfectant in high concentration.

Depending on the antimicrobial effectiveness expected from chemical agents under set conditions, disinfectants can be classified as "high level" (having sterilization activity) such as peroxides and aldehydes, "intermediate level" (which inactivate *Mycobacterium tuberculosis* and the most resistant types of viruses but not spores) such as alcohol and hypochlorite or "low level" (reduction of bioburden) such as phenol and quaternary ammonium compounds (QACs) (Gamage, 2003; Rutala et al., 1998).

Hydrogen peroxide works by the production of

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Table 1. Antiseptics and disinfectants used and their dilution and composition (Sheraba et al., 2012).

Biocidal solution	Purpose	Use dilution	Composition
Sanigel	Antiseptic	75%	Ethanol 62%, deionized water, glycerin, perfume oil, PEG-9, Dimethicone, Isostearate and acrylic polymer (Formuln, Egypt).
Hospidermin	Antiseptic	75%	42.6 g ethanol 96%, 3 g potassium thiocyanate, 0.1 g 5-Chloro-2-hydroxy-benzoicacid, Butan-2-one, colouring additives: E 124 and E 110 and Purified water, in 100g (Lysoform, Berlin, Germany).
Alcohol	Antiseptic	75%	95% ethanol (United Company for Chemicals, Egypt).
AHD 2000	Antiseptic	75%	79.6% ethanol, lactic acid, fragrance, water, macrogolglycerolcocoate (Lysoform, Berlin, Germany).
Sanipine	Disinfectant	1:20	Sodium lauryl sulphate, QAC, solvents with pine smell (Formuln, Egypt).
Sanismell	Disinfectant	1:20	Sodium lauryl sulphate, QAC, solvents with fresh smell (Formuln, Egypt).
Lysoformin 3000	Disinfectant	1%	7.5 g glyoxal, 9.5 g glutaraldehyde, 9.6 g didecyl dimethyl ammonium chloride, in 100 g (Lysoform, Berlin, Germany).
Trichlorol	Disinfectant	0.75%	80 g sodium tosylchloramide. $3H_2O$, sodium lauryl Sulphate, sodium chloride, fragrance, in 100 g (Lysoform, Berlin, Germany).
H_2O_2 25%	Disinfectant	3%	Hydrogen peroxide 25% (Roam Chene, Belgium)
Chlorax	Disinfectant	1:16	Sodium hypochlorite, surfactant (Chlorax, Egypt).

destructive hydroxyl free radicals that can attack membrane lipids, DNA, and other essential cell components. Formaldehyde acts by alkylating the amino and sulfhydryl groups of proteins and ring nitrogen atoms of purine bases (Favero and Bond,1991). Glutaraldehyde interacts with amino acids within proteins of microorganisms (McDonnel and Russell, 1999). Chlorine acts as an oxidizing agent (Russel et al., 1982). Alcohol inactivates microorganisms by denaturation of proteins (Fraise, 1999). Phenol, in high concentrations, acts as a gross protoplasmic poison, in low concentrations cause bacterial death by the inactivation of essential enzyme systems and leakage of essential metabolites from the cell wall while QACs have been attributed to the inactivation of energy-producing enzymes, denaturation of essential cell proteins, and disruption of the cell membrane (Rutala et al., 2002).

To reach the level of hygiene required in a health care facility and before the application of biocides, it is necessary to standardize the use of any biocide and select the test organism that is mostly prevalent or common to the material or environment upon which the particular biocide is to be applied (Croshaw, 1981). In a previous study carried out by the same authors in the same facility, the *Staphylococci* spp. represented the most common isolate that was detected from an environmental monitoring programme with *Staphylococcus hominis* representing 51% of all *Staphylococcci* isolates followed by *Stapyhlococccus epidermidis* (14%)*, Stapyhlococccus haemolyticus* (12%) and *Micrococcus* spp. (Sheraba et al., 2010).

The aim of the present study was to test the bactericidal, sporicidal and fungicidal activity of the main antiseptics and disinfectants that are commonly used in the main vaccine production facility in Egypt. The standard microorganisms recommended by the pharmacopeia were used, in addition to the main bacterial isolates that were previously identified in an environmental monitoring program to be the main contaminants likely to be present in the same facility.

MATERIALS AND METHODS

A list of all disinfectants and antiseptics used with their composition and dilutions is listed in Table 1. A list of all neutralizers with their exact composition is listed in Table 2. All culture media were from Bacto, France.

Bacterial and fungal strains

Staphylococcus aureus ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404 and *Bacillus subtilis* ATCC 6633 were selected as index organisms representing Gram-positive and Gram-negative bacteria, fungi, and spore forming bacteria respectively. *S. hominis, S. epidermidis, S. haemolyticus* and *Micrococcus* spp. isolated from an environmental monitoring program previously carried out in the same facility were also used in the tests.

Preparation of working cultures of bacterial, fungal and spore suspension

According to European standard EN 12353 (Jette et al., 1995), test organisms were subcultured from the stock culture by streaking into trypticase soy agar (TSA) media , and incubated at 36°C for bacteria or into sabouraud dextrose agar (SDA) media, and incubated at 20-25°C for fungi (in the case of *Aspergillus*). After 24 h, a second subculture was prepared from the first one in the same way and incubated again. The test organisms of the second subculture were washed off with 10 ml of a diluent containing 0.1%

Table 2. List of neutralizers used (Sheraba et al., 2012).

Neutralizer	Concentration
A- 1% Physiological peptone water	
Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	3.5 g
Monopotassium Phosphate	1.5 g
0.9% Saline	1000 ml
B- 0.6 % Sodium thiosulphate in 1% physiological peptone water	
C- Phosphate Buffer Saline	
D- 0.1% Tween 80 in 1% physiological peptone water	
E- Dey-Engley Broth	
Sodium thioglycolate	1g/l
Sodium thiosulfate	6 g/l
Sodium bisulfite	2.5 g/l
Polysorbate 80	5g/l
Lecithin (soybean)	7 g/l
Tryptone	5 g/l
Yeast extract	2.5 g/l
Glucose	10 g/l
Distilled water	1000 ml

(w/v) tryptone and 0.85 % (w/v) sodium chloride; adjusted to McFarland standard of 2 units (1.5-5X10^9 CFU/ml). For *Aspergillus niger*, a subculture was grown on SDA and incubated at 20-25°C for 7-14 days then mycelial mats were harvested from the agar surface of the working culture, homogenized with sterile glass beads in 1% physiological peptone and filtered through sterile cotton gauze to remove hyphae then suspended in 1% physiological peptone with 0.1% Tween 80 and adjusted to McFarland standard of 2 units (1.5-5X10^9 CFU/ml). Spore cultures were developed for a period of 3-8 weeks in a sporulation medium (15 g/L Peptone, 3 g/L yeast extract; 6.0 g/L NaCl, 1.0 g/L D (+)-glucose; 0.1 g/L Manganese sulphate) at 37°C then they were harvested, centrifuged (4 times at 1935 × *g*/ 30 min), heat-shocked 80°C/10 min and the spore suspension was adjusted to McFarland standard of 2 units (1.5-5X10^9 CFU/ml).

Preparation of the biocide dilutions

The procedure was done according to Salo and Wirtanen (2005). Each of the biocide to be tested (mentioned in Table 1) was diluted in sterile distilled water according to the recommendations of the manufacturer. The initial dilution was termed the "use-dilution" with two further dilutions prepared (one above and one below the use-dilution) as shown in Tables 3 to 8. The preparations were filtered through 0.22 µm membrane filter, pH was adjusted to 6.8-7.0, and all preparations were made fresh prior to testing.

Challenge test procedure

Quantitative suspension test was used according to Hernandez et al. (2003) as follows; for each microorganism used, 0.1 ml of bacterial or fungal suspension (1X10^9CFU/ml) was added separately to 10 ml biocide at room temperature. Controls contained 10 ml diluent instead of disinfectant or antiseptic. After contact times (5, 10, 20 and 30 min for disinfectant) or (30 s, 1 min, 2 min and 5 min for antiseptic), 1 ml was added to 9 ml neutralizing solution in order to terminate the activity of the biocide without interfering with survivor growth. The neutralizers chosen for each biocide with each test organism were previously tested and determined in a previous study that was carried out in the same facility as part of the same project (Sheraba et al., 2012). Serial dilutions were prepared (1:10, 1:10^2, 1:10^3, 1:10^4, and 1:10^5) and 0.1 ml was taken from each serial dilution tube and plated onto TSA with 0.5% glucose by the spread-plate technique with sterile glass spatula. Plates were incubated for 24-48 h at 37°C for bacteria, spores and bacterial environmental isolates and into SDA and incubated for 3-5 days at 22.5°C for fungi and yeast. For each biocide, the test was repeated three times, and then average colonies count were enumerated and expressed as colony-forming units per millilitre.

Determination of activity

The logarithmic reduction factor (RF) was calculated as the expression of the biocide efficacy, according to the following formula (Russell et al., 1982; James, 1999; MerapÖzalp et al., 2007):

RF= log Nc - log Nd

RF is the Logarithmic reduction factor, Nc is bacterial colony

Table 3. Evaluation of bactericidal activity of test antiseptics

Chemical agent name	Initial inoculum	Dilution (%)	Contact time			
			30"	60"	120"	300"
Alcohol	$1X10^8$ cfu/ml	50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++
AHD 2000		50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++
Sanigel		50	+	++	+++	+++
		75	++	++	+++	+++
		95	++	+++	+++	+++
Hospidermin		50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++

Table 4. Evaluation of bactericidal activity of test disinfectants

Chemical agent name	Initial inoculum	Dilution	Contact time			
			5'	10'	20'	30'
Lysoformin 3000	$1X10^8$ cfu/ml	0.5%	+++	+++	+++	+++
		1%	+++	+++	+++	+++
		2%	+++	+++	+++	+++
Trichlorol		0.5%	+++	+++	+++	+++
		0.75%	+++	+++	+++	+++
		3%	+++	+++	+++	+++
Chlorax		1:32	+++	+++	+++	+++
		1:16	+++	+++	+++	+++
		1:8	+++	+++	+++	+++
H_2O_2 25%		2%	+++	+++	+++	+++
		3%	+++	+++	+++	+++
		5%	+++	+++	+++	+++
Sanipine		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	+
Sanismell		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	+

number from control plates and Nd is the Bacterial colony number after contact with biocide.

Highly effective (+++) = Log_{10} reductions of value >5.
Effective (++) = Log_{10} reductions of value = 5.
Less effective (+) = Log_{10} reductions of values between 1-5.
Ineffective: (-) = Log_{10} reductions of 1.

RESULTS

Evaluation of bactericidal activity

As shown in Table 3, the antiseptics: Alcohol, AHD 2000 and Hospidermin were highly effective against both Gram-positive and Gram-negative bacteria at all concentrations and contact times tested. Also, Sanigel was highly effective at concentration 50, 75 and 95% at 60 s, 120 s and 300 s contact times, less effective at concentration 50% in 30 s and effective at 50% at 60 s, at 75% at 30 s, 60 s and at 95% at 30 s. For the disinfectants tested as shown in Table 4, Lysoformin 3000, Chlorax, Trichlorol and H_2O_2 25% were highly effective against both Gram-positive and Gram-negative bacteria at all concentrations and contact time tested, while Sanipine and Sanismell showed only limited activity

Table 5. Evaluation of fungicidal activity of test antiseptics.

Chemical agent name	Initial inoculum	Dilution (%)	Contact time			
			30"	60"	120"	300"
Alcohol	$1X10^8$ cfu/ml	50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++
AHD 2000		50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++
Sanigel		50	+	+	++	++
		75	++	++	++	++
		95	+++	+++	+++	+++
Hospidermin		50	+++	+++	+++	+++
		75	+++	+++	+++	+++
		95	+++	+++	+++	+++

Table 6. Evaluation of fungicidal activity of test disinfectants.

Chemical agent name	Initial inoculum	Dilution	Contact time			
			5'	10'	20'	30'
Lysoformin 3000	$1X10^8$ cfu/ml	0.5%	+++	+++	+++	+++
		1%	+++	+++	+++	+++
		2%	+++	+++	+++	+++
Trichlorol		0.5%	+	+	+	++
		0.75%	+	++	++	+++
		3%	++	++	+++	+++
Chlorax		1:32	+++	+++	+++	+++
		1:16	+++	+++	+++	+++
		1:8	+++	+++	+++	+++
H_2O_2 25%		2%	+++	+++	+++	+++
		3%	+++	+++	+++	+++
		5%	+++	+++	+++	+++
Sanismel		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	+
Sanipane		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	+

at 1:15 for 30 min.

Evaluation of Fungicidal activity

As shown in Table 5, the antiseptics, Alcohol, AHD 2000 and Hospidermin were highly effective against fungi and yeast at all concentrations and contact times tested. In contrast, Sanigel was less effective at concentration 50% at 30 and 60 scontact times, effective at concentration 75% and highly effective at 95%. For the disinfectants tested as shown in Table 6, Lysoformin 3000, Chlorax and H_2O_2 25% were the most potent disenfectants against the fungi tested (*A. niger* and *C. albicans*) followed by Trichlorol, while Sanipine and Sanismell performed less effectively.

Evaluation of Sporicidal activity

All antiseptics mentioned were found to be ineffective against spores of *B. subtilis* (Table 7). For the tested

disinfectants, H_2O_2 25% and Chlorax were found to be highly effective against *B. subtilis* spores at concentration of 2, 3, 5% and 1:8 and 1:16 respectively at 20 and 30 min contact time, while Lysoformin 3000 was found to be less effective at concentration 1 and 2% at 30 min. Trichlorol was found to be less effective at 3% for 10, 20, 30 min contact time, while Sanipine and Sanismell were shown to be ineffective (Table 8).

DISCUSSION

In this study, quantitative suspension test was performed to evaluate the bactericidal, fungicidal and sporicidal activity of four chemical antiseptics and six chemical disinfectants at various concentrations, at defined contact times, in the absence of interfering substances. Concerning the bactericidal and fungicidal efficiencies of the tested antiseptics, the results indicate that three of the alcohol based biocides (Alcohol, AHD 2000 and Hospidermin) reduced the viable titers of the suspended vegetative bacteria and fungi by more than 5 log values even at a concentration of 50% and within 30 s. The fourth alcohol based biocide, Sanigel, reached the same efficacy but at the longer contact time of 120 s. Similar findings have been reported by other groups showing that alcohol based antiseptics have a very rapid and effective antimicrobial action that exceeds any other antiseptic at concentration 50-90% for shorter contact times against bacteria and fungi (Van Klingeren et al., 1998; Vieira et al., 2005; Jeng and Severin, 1998).

Among the six disinfectants tested, only the aldehyde-based disinfectant (Lysoformin 3000), Chlorine-based: (Chlorax and Trichlorol) and Hydrogen peroxide had an excellent killing activity within 5 min against the suspended vegetative bacteria and fungi at the recommended concentrations. These findings are similar to another study, which showed that 2% Lysoformin 3000 at 30 min was enough for bactericidal and fungicidal activity (Aksen et al., 2004). It was reported that all chlorine-based disinfectants were significantly more effective in killing bacteria and fungi in 5 min and the efficiency was generally increased when the contact time was prolonged from 5 to 15 min (Van Klingeren et al., 1998; Özalp et al., 2007; Salo and Wirtanen, 2005). Other groups reported that 3-25% hydrogen peroxide was able to inactivate bacteria and fungi by > 6 $\log_{10}$ reduction in a contact time of 5 min, while, Sanipine and Sanismel were effective only at 1:15 for 30 min requiring prolonged exposure time or greater concentration (Russell, 1998; Alfa and Jackson, 2001; Sattar et al., 2002). Quaternary ammonium compounds gained popularity due to its good antibacterial and antifungal at relatively low concentration within shorter contact time (Russell, 1998; Fraise, 1999).

The principal of evaluation of sporicidal activity was the same as for determination of bactericidal activity; the main difference was to achieve sporulation (Russell, 1998). Our results indicate that all alcohol based antiseptics were found to be nonsporicidal. Similar findings have been reported by other groups for these agents (Fraise, 1999; Gupta et al., 2007). Hydrogen peroxide (25%) was found to the more rapid and effective in killing spores with all its recommended concentrations (Coates, 1996; Jose-Luis Sagripanti and Bonifacino, 1996) which agrees with our findings. Chlorax showed high killing effect at concentration1:8 and 1:16 for 20-30 min, however, Trichlorol requires higher concentration to achieve the same effect of killing as Chlorax which agrees with the study done by Russel in 1998 (Russell, 1998). In contrast, 30 min of 2% Lysoformin 3000 was found to be effective against bacterial spores as reported (Aksen et al., 2004; Holton et al., 1995) who noted that glutaraldehyde based disinfectants have slow sporicidal effect while sanismel and Sanipine were found to be nonsporicidal.

Most previous reports have only analyzed the effect of biocides against reference strains from ATCC (Jang et al., 2008; Sagripanti and Bonifacino, 1996; Crowe et al., 2007). Susceptibility of environmental isolates to biocides is now attracting special attention since ATCC strains that are laboratory adapted, may not be good predictors for the susceptibility of strains extracted from environments. It was interesting that environmental isolates as *Micrococcus* spp., *S. hominis*, *S. haemolyticus* and *S. epidermidis* also showed the same susceptibility as ATCC strains.

Conclusion

From the tested biocides, hydrogen peroxide showed the strongest effect as it had bactericidal, fungicidal and sporicidal activity. Alcohol or chlorine based compounds, and gluteraldehyde showed bactericidal and fungicidal activity but no spirocidal activity except for some used dilutions at certain contact times in case of chlorine based compounds and gluteraldehyde. Quaternary ammonium compounds had the weakest activity in all tests.

It is essential to constantly monitor the activity of biocides that are frequently used in any health care settings as represented in this work by the vaccine production facility where this work was done. The efficacy of the biocides should be tested with index micro-organisms as well as common isolates identified in environmental monitoring programs and known to be possibly present in that particular location.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Hamdalla Hafez Zedan, chairman of the Egyptian Company for the

Table 7. Evaluation of sporicidal activity of test antiseptics.

Chemical agent name	Initial inoculum	Dilutions (%)	Contact time			
			30"	60"	120"	300"
Alcohol	$1X10^8$ cfu/ml	50	-	-	-	-
		75	-	-	-	-
		95	-	-	-	-
AHD 2000		50	-	-	-	-
		75	-	-	-	-
		95	-	-	-	-
Sanigel		50	-	-	-	-
		75	-	-	-	-
		95	-	-	-	-
Hospidermin		50	-	-	-	-
		75	-	-	-	-
		95	-	-	-	-

Table 8. Evaluation of sporicidal activity of test disinfectants.

Chemical agent name	Initial inoculum	Dilution	Contact time			
			5'	10'	20'	30'
Lysoformin 3000	$1X10^8$ cfu/ml	0.5%	+	+	+	+
		1%	+	+	+	++
		2%	+	+	++	++
Trichlorol		0.5%	-	-	-	+
		0.75%	-	-	+	+
		3%	-	+	+	+
Chlorax		1:32	+	++	++	+++
		1:16	++	++	+++	+++
		1:8	++	+++	+++	+++
H_2O_2 25%		2%	++	++	+++	+++
		3%	++	+++	+++	+++
		5%	+++	+++	+++	+++
Sanismel		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	-
Sanipane		1:30	-	-	-	-
		1:20	-	-	-	-
		1:15	-	-	-	-

production of Vaccines, Sera and Drugs (EGYVAC), for his continuous support and help in carrying out the study.

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Vol. 8(5), pp. 425-431, 29 January, 2014
DOI: 10.5897/AJMR2013.6319
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Microbiological contamination of surfaces in fish industry

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Accepted 20 January, 2014

Three hundred and forty (340) samples of surfaces from equipment (skinning machines), utensils (polyethylene cutting boards, polypropylene cases, baskets, and trays, plastic material used to cover the trays, packaging tanks, knives, and stainless steel sorting and packaging tables), and gloves used by handlers in fish industries, collected with swabs in August 2010 and August 2011, were evaluated. In each period, five different collections were made on different days in both the early morning and early afternoon. Counts of aerobic mesophiles and total coliforms were performed and the presence of thermotolerant coliforms was investigated. All samples collected in the afternoon shift, in either collection periods, showed significantly higher contamination by aerobic mesophiles compared to the morning shift ($p<0.05$). 50.0 and 81.8% of the equipment and utensils analyzed in the first and second collection, respectively, were within the recommendations by the Pan American Health Organization (PAHO) regardless of the work shift. The gloves in the first collection period had aerobic mesophile count above 4 log UFC/glove in 76.7% of the samples and in only 21.7% in the second collection. Although surface contamination decreased, corrective measures still must be enforced and the employees must be oriented regarding the importance of hygienization.

Key words: Hygienization, mesophiles, equipment, utensils, handlers.

INTRODUCTION

Since fish is a food with high nutritional value with pH close to neutral and high water activity, it is very susceptible to spoilage. Besides its autochthonous microbiota, located mainly in the intestines, gills, and surface mucus, they may also be contaminated by spoilage and pathogenic bacteria coming not only from the aquatic environment, but also from inappropriate processing and storage (Ghaly et al., 2010; Mol and Tosun, 2011).

Poor hygienization processes of surfaces that make contact with fish during all production stages are also a crucial factor for the quality of the final product (Kusumaningrum et al., 2003; Temelli et al., 2006; Mol and Tosun, 2011). Dirt particles and microorganisms that fail to be removed by correct hygienization procedures may start adhesion processes and lead to the formation of biofilms (Andrade, 2008; Salustiano et al., 2010).

Microorganism contamination of equipment and utensils is a risk factor in the food industry, therefore the choice of material they are made of must be based on their mechanical and anti-corrosive properties and on the ease of hygienization (Silva et al., 2003; Fuster-Valls et

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Table 1. Surfaces analyzed number of samples, type of collection and the total area sampled for microbiological evaluation in fish industry.

Parameter	Analyzed surfaces	n	Collection	Total sampled area (cm^2)
Equipment	Skinning machine 1 (inox)	5	Unit	100
	Skinning machine 2 (inox)	5	Unit	100
Utensils	Baskets	5	Lot*	500
	PVC films	5	Lot	500
	Cutting boards	5	Lot	500
	Trays	5	Lot	500
	Cases	5	Lot	500
	Sorting tables (inox)	5	Unit	200
	Packaging tables (inox)	5	Unit	200
	Packaging tanks (inox)	5	Unit	200
	Knives (inox)	5	Lot	Surface
Gloves	Latex Gloves	30	Pair	Surface

n, total samples per shift; *one lot is a collection of 5 units.

al., 2008). Some studies have reported high incidence of microorganisms in equipment and utensils in food-processing areas caused by failures in employing correct hygienization techniques, which results in serious public health or economic issues (Temelli et al., 2006; Oliveira et al., 2008, Kahraman et al., 2010).

The Brazilian legislation does not set microbiological parameters for surfaces of equipment and utensils used in food processing, as well as for the handlers' hands. The standards of the American Public Health Association (APHA) consider equipment and utensils clean if they have less than 2 log CFU/utensil or 0.3 log CFU/cm^2 (Evancho et al., 2001).

However, developing countries have difficulties in adapting industries to the American standards, so the Pan American Health Organization (PAHO) recommends counts up to 1.7 log CFU/cm^2 or 2 log CFU/utensil for aerobic mesophiles and absence of thermotolerant coliforms mainly due to the ambient temperatures in these countries (Cardoso et al., 2011).

Another factor that must also be taken into account in the food production chain is the handlers, who must be trained in Good Manufacturing Practices (GMPs) and have adequate personal hygiene (Brasil, 2009). Otherwise, they may carry pathogens, which is often reported as the cause of foodborne diseases (Rosas and Reys, 2008; Dias et al., 2012). Andrade (2008) set two count ranges that could serve as a guideline to define hygienic-sanitary hand conditions: range 1 (up to 3 log) and range 2 (between 3 and 4 log), expressed as CFU/hand for aerobic mesophiles and total coliforms.

Surface cleaning and disinfection procedures, despite being essential for good-quality and safe foods, are often not a priority. Not always is the cost-benefit relation of these practices acknowledged since their results are not easily measured in terms of economic gains (Aarnisalo et al., 2006).

The goal of this study was to evaluate the levels of microbiological contamination of several surfaces (equipment, utensils, and gloves) before they were used in the fish processing plant, aiming to verify the efficiency of the Standard Operating Hygiene Procedures (SOHP) applied and the influence of implementing GMPs in the plant.

MATERIALS AND METHODS

Characteristics of the fish processing plant

The fish processing facility is located in the northeast of the state of Pará, Brazil, and was in the process of implementing GMPs. The industry has 129 employees and can process about 10 t/day of fish. It produces several frozen products such as whole eviscerated fish, fish fillets, and steaks of different species, which are marketed across Brazil and exported to the United States.

Sample collection

Equipment, utensils and non-disposable rubber gloves used in the fish processing were analyzed (Table 1). The sampling of the surfaces was performed in two periods: In August 2010, at the beginning of GMP implementation, however before employee training began, and in August 2011, when the plant was already in the final process of GMP implementation. In each period, five collections were made on different days and in both shifts, early in the morning and early in the afternoon, before the surfaces were used in the processing.

These shifts were established based on the time of hygienization of most surfaces, which was performed twice a day, between 11 a.m. and 13 p.m. (lunch) and after the end of the working hours (6 p.m.). In each collection, the surfaces were analyzed individually (two skinning machines, two tables, one tank, and six pairs of gloves) or in batches of five units per surface (baskets, cutting boards, cases, PVC films, and knives). 340 samples were analyzed in total, 170 for each collection period.

During the lunch break, in both collection periods, the skinning machines and the fixed utensils in the production area such as tables and tanks were only washed with water jets. The use of detergent and sanitizer was conditioned to the absence of raw

material and/or products in the processing line to avoid chemical contamination. The other utensils, despite having specific areas for hygienization, underwent only cleaning in the first collection period. However, in the second collection period, the use of sanitizer was verified, albeit sometimes diluted incorrectly. Only by the end of the working hours, in both collection periods, did all the equipment and utensils underwent the cleaning and sanitization steps. However, most times the equipment was not taken apart.

The samples from equipment and utensils were collected with swabs following the procedure proposed by the American Public Health Association (Evancho et al., 2001). After being dipped in a diluent solution (0.1% sterile peptone water), sterile cotton swabs were rubbed three times on an area not smaller than 100 cm^2 or on all the surface area that touched the food.

On the gloves, the analysis area was the surface of the palm and the edges starting from the wrists. In an angle, the swab was rubbed with circular motion from the lower part of the palm until the tip of the fingers and back to the wrist, a procedure that was repeated three times for each finger. The collection on the edges used a back-and-forth motion, starting from one side of the hand where the wrist begins, going between the fingers, and finishing at the wrist on the other side of the hand (Andrade, 2008).

After this sampling, the swabs were placed in test tubes containing 10 ml of sterile peptone water with 1 of 0.25% sodium thiosulfate. The tubes were then capped, identified, and immediately taken to the laboratory under refrigeration for the analyses.

During the collection, the processing's routine, employee behavior, and the Standard Operating Hygiene Procedures (SOHP) applied were followed. This task was performed through observations at the site, by checking paperwork, and through information provided by the employees and owners.

Microbiological analyses

After appropriate decimal dilutions (down to 10-4) with sterile 0.1% peptone water, the samples were plated in Plate Count Agar (PCA - OXOID CM 325) for the count of total aerobic mesophiles, and in Violet Red Bile Glucose Agar (VRBGA, OXOID CM 485) for the enumeration of total coliforms with later confirmation of the presence or absence of thermotolerant coliforms. All the analyses were performed in triplicate and followed the methodology described in the Compendium of Methods for the Microbiological Examination of Foods (Downes and Ito, 2001).

The results of the Colony-Forming Units (CFU) by cm^2 of the surface, or CFU/surface, were converted into logarithms and compared with the recommendations of the Pan American Health Organization (PAHO) (Cardoso et al., 2011).

Statistical analysis

The values of the average counts of aerobic mesophiles (log CFU/cm^2 or log CFU/surface) underwent analysis of variance (ANOVA) and Tukey's test using the software Statistica® version 7.0 to check whether there was a significant difference ($p \leq 0.05$) between the work shifts and periods analyzed.

RESULTS AND DISCUSSION

Evaluation of the application of the SOHP and microbiological analyses of the fish processing plant's equipment and utensils

The variation in the aerobic mesophile counts in the various collections from the same surface (Table 2) shows that there is no standardization in the hygienization processes in the processing plant. The failure in fully following the SOHPs, at all times of hygienization, may lead to a variation in microbial counts, which may then compromise the hygienic-sanitary quality of foods (Kahraman et al., 2010; Salustiano et al., 2010).

The high microbial load found in some of the equipment and utensils analyzed (Table 2) and the significantly higher levels of aerobic mesophile contamination in all samples collected in the afternoon shift, in both collection periods, are attributed to inefficient cleaning. High microbial counts in food-processing plant surfaces indicate the inefficient application of the SOHPs, risk of cross-contamination, possibility of biofilm formation, and possible presence of pathogens (Aarnisalo et al., 2006; Lequette et al., 2010). Foods in contact with contaminated surfaces may have their microbiological quality compromised, especially if they're consumed raw or if the thermal treatment is not adequate for inactivating vegetative cells or bacterial toxins that might be present (Temelli et al., 2006; Jha et al., 2010).

The contamination of foods by sessile microbial cells has already been show in several studies. Salustiano et al. (2010) assessed post-pasteurization recontamination of milk by *Bacillus cereus* using automated ribotyping. Seven ribogroups were identified and the same ribogroup was isolated from four surfaces and milk samples, suggesting the surfaces are repositories of that species. Ravishankar et al. (2010), while studying the occurrence of cross-contamination, showed that *Salmonella enterica* serovar Newport, present in poultry, was able to contaminate the stainless steel knife and polyethylene cutting board, being then transferred to lettuce leaves.

Out of all the samples from the surfaces of equipment and utensils analyzed, irrespective of the shift, 50.0 and 81.8% were within the limits recommended by the PAHO in the first and second collection periods, respectively. The increase in conformity seen in the second period may be mainly attributed to the GMPs that were being implemented in the fish processing plant and to the training of the handlers, especially in hygienization procedures. Hwang et al. (2011), while evaluating several surfaces of different fish-processing areas in Taiwan, found that the contamination level was lower ($p<0.05$) in the industries that had already implemented quality management tools.

In the first period, 31.8% of the samples from equipment and utensils were out of the PAHO standard for aerobic mesophiles and thermotolerant coliforms, while 18.2% were not within the limit established for aerobic mesophiles. However, in the second period, only 9.1% of the samples had mesophiles as the main microorganisms responsible for contamination and 9.1% were not within the limits for aerobic mesophiles and thermotolerant coliforms.

Among the samples out of the standard, 73.3% were detected in the afternoon shift due to the lack of

Table 2. Mesophile count and survey of thermotolerant coliforms in surfaces of a fish industry from collections in August 2010 and August 2011 in two shifts (morning and afternoon).

Surfaces (equipment and utensils)	August 2010				August 2011			
	Aerobic mesophiles[1] (log CFU/cm^2)		Coliforms[2] (P/A)[3]		Aerobic mesophiles[1] (log CFU/cm^2)		Coliforms[2] (P/A)[3]	
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon
Skinning machine 1	2.52±0.05[a]	4.14±0.07[b]	A	P	1.35±0.19[a]	2.99±0.05[b]	A	P
Skinning machine 2	2.54±0.06[a]	4.17±0.08[b]	A	P	1.41±0.13[a]	3.01± 0.09[b]	A	P
Baskets	1.49±0.09[a]	5.32±0.07[b]	A	P	1.42±0.12[a]	1.81±0.03[b]	A	A
PVC films	1.56±0.08[a]	4.98±0.02[b]	A	P	1.08±0.22[a]	1.59±0.09[b]	A	A
Cutting boards	1.25±0.13[a]	1.82±0.07[b]	A	A	1.30±0.27[a]	1.67±0.07[b]	A	A
Trays	0.99±0.35[a]	1.76±0.04[b]	A	A	0.95±0.28[a]	1.61±0.06[b]	A	A
Cases	1.25±0.36[a]	1.85±0.15[b]	A	A	0.34±0.23[a]	1.08±0.22[b]	A	A
Sorting tables	0.31±0.33[a]	0.84±0.24[b]	A	A	0.30±0.30[a]	0.85±0.08[b]	A	A
Packaging tables	0.57±0.33[a]	1.35±0.13[b]	A	A	0.46±0.18[a]	1.26±0.15[b]	A	A
Packaging tanks	0.36±0.39[a]	0.99±0.14[b]	A	A	0.33±0.32[a]	0.98±0.10[b]	A	A
Knives[3]	3.65±0.05[a]	3.87±0.03[b]	P	P	1.54±0.06[a]	2.03±0.16[b]	A	A

[1]Average±standard deviation (n=5). [2]Thermotolerant coliforms. [3]P, presence ; A , absence. [4]CFU/utensil. [b]Different small letters in the same line, in each year of collection, means the results were significantly different ($p<0.05$).

or inappropriate sanitization.

Several authors (Sneed et al., 2004; Oliveira et al., 2008; Wang et al., 2010), when assessing equipment and utensils in businesses, detected 100% of samples above the PAHO guidelines and highlighted that the inappropriate hygiene and sanitation conditions of the surfaces analyzed are responsible for an increase in spoilage and pathogenic microorganisms in the final products. According to Aantrekker et al. (2003), when the contribution of air contamination can be quantified, its importance can be determined in an overall risk assessment by comparing air contamination to other sources (initial contamination and other contamination routes).

Among the surfaces with high contamination level, the skinning machines 1 and 2 stood out for having high counts of aerobic mesophiles ranging between 2 and 4 log CFU/cm^2 and from 1 to 3 log CFU/cm^2 in the first and second periods, respectively. However, only in the samples collected in the afternoon shift, in either collection period, was the presence of thermotolerant coliforms detected (Table 2). One of the reasons of this contamination is the inappropriate design of the equipment, which makes cleaning hard and hinders the action of the sanitizing agent that makes it necessary to hygienize the equipment more often using more aggressive chemicals, which does not guarantee the safety in food production (Lelieveld et al., 2003). Thus, the equipment must be designed in a way to make cleaning, sanitization, inspection, and maintenance easy (Aarnisalo et al., 2006).

Kahraman et al. (2010) reported that for appropriate hygienization, the equipment must be disassembled prior to cleaning. Several studies have also linked high levels of contamination by aerobic mesophiles (2 to 5 log CFU/cm^2) and the presence of thermotolerant coliforms found in equipment of different food-processing areas to hardships in carrying out cleaning due to the difficulty in disassembling them, which causes accumulation of residues (Oliveira et al., 2008; Keeratipibul et al., 2009; Cardoso et al., 2011).

Among the utensils analyzed, it can be seen in Table 2 that only the knives had high contamination by aerobic mesophiles (>1.7 log CFU/utensil) in either shift in the first collection period and only in the afternoon shift in the second period. The presence of thermotolerant

coliforms was detected only in both shifts of the first period. In the plant analyzed, each handler is responsible for hygienizing the knife used. The results found, despite the significant ($p<0.05$) reduction found between the two collection periods, suggest that the handlers were still not fully aware of the importance of appropriate hygienization, which prevents contamination sites. Therefore, the plant's GMP trainings must be ongoing. Çetin et al. (2006), when evaluating several utensils used in a meat-processing plant, also found high levels of aerobic mesophilic bacteria (up to 2 log CFU/cm^2) on the knives and highlighted that this result means the knives may pose a real threat if associated to the presence of pathogens.

On the baskets, PVC films, cutting boards, trays, and cases, the levels of aerobic mesophiles were also high (>1.7 log CFU/cm^2) in the afternoon shift of the first collection period (Table 2), likely due to the lack of sanitization. In the second period, these utensils were already being sanitized in the afternoon shift, although the baskets still had high microbial counts perhaps since the holes made hygienization harder. The presence of thermotolerant coliforms was only seen on the baskets and PVC films in the afternoon shift during the first collection period. It is important to note that the plastic sheets used to cover the trays were very worn out, making hygienization harder (Kusumaningrum et al., 2003). That is why these sheets had all been replaced in the second collection period, which must also have contributed to reducing the microbial counts in this period (Table 2).

Low counts of aerobic mesophiles (<1.5 log CFU/cm^2) and no thermotolerant coliforms were detected on the tables and in the tank (Table 2) even in the shifts when they were only washed with water jets. These results may be related to the type of material (stainless steel) and to the pristine conditions of these surfaces, which allowed more appropriate hygienization or cleaning. Materials such as stainless steel allow for more efficient hygienization, especially if appropriate chemicals are used (Fuster-Valls et al., 2008). Cabeça et al. (2006) found a reduction in the number of *L. monocytogenes* cells adhering to the surface of stainless steel after the treatment with different sanitizers (iodine, biguanide, quaternary ammonium compounds, peracetic acid, and sodium hypochlorite).

Microbiological analyses of gloves

On the non-disposable rubber gloves used by all handlers in the processing line, counts of aerobic mesophiles and total coliforms ranging from 3.3 to 6.9 log CFU/glove and 2.2 to 3.8 CFU/glove were found, respectively, in the first collection period. In the second period, the values ranged from 2.1 to 5.9 log CFU/glove and from 1.0 to 3.7 log CFU/glove for mesophiles and coliforms, respectively.

Oliveira et al. (2008), when assessing the hands of handlers working directly with meat grinders in five business facilities, detected aerobic mesophiles ranging from 4.4 to 6.8 log CFU/hand and thermotolerant coliforms from 1.2 to 3.7 CFU/hand. According to those authors, these results suggest inappropriate hygienization and may be a source of meat contamination after grinding and handling.

Rosas and Reys (2008) while observing the personal hygiene practices in a fish processing plant noticed that the handlers often did not wash their hand before beginning work in the production area. Thus, they claim that training is crucial to improve handler hygiene practices and that supervision must be constant to assure the correct application of the cleaning and sanitization procedures so as to avoid cross-contamination.

Between the collection periods, only in the first period was a significant difference ($p<0.05$) found between the mesophile counts. Nevertheless, the number of mesophiles and coliforms, regardless of the shift, has a significant reduction ($p<0.05$) between the two periods (Figure 1), once again showing the importance of implementing GMPs. Dias et al. (2012) related the decrease in coliform count, from 5.8 to 1.2 log CFU/hand, in handlers' hands in a cheese processing industry to the implementtation of GMPs. According to those authors, the changes carried out were appropriate to improve the hygiene practices adopted in the industry, which will certainly influence the production of better quality and safer cheese.

According to criterion suggested by Andrade (2008), in the first collection period only 23.3% of the glove samples had counts of aerobic mesophiles within range 2, which is the acceptable limit for microbial count. However, that represents a warning that the hygienization procedure must be controlled; the other 76.7% of samples were above 4 log CFU/glove, indicating a poor hygienization process. In the second period, 20% of the samples were within range 1, 58.3% were within range 2 and 21.7% were above 4 log CFU/glove. The coliform count in both periods was below 4 log CFU/glove. Microbial counts above 4 log CFU/hand highlight the importance of handlers as potential food contamination agents (Dias et al., 2012).

Despite the reduction in contamination level found, glove hygienization, which is a responsibility of the handlers themselves in the industry analyzed, still needs to be improved since they may be sources of spoilage and pathogenic microorganism contamination (Rosas and Reys, 2008; Dias et al., 2012). Such contamination must be reduced or eliminated in order to prevent its introduction in the foods and, consequently, impairing its commercial life or posing potential risks to consumers.

According to Aycicek et al., (2006), seeing apparently clean surfaces may lead to error and give a false feeling of safety. The microbiological trials do not prevent bacteria from entering the industry, but allow keeping an

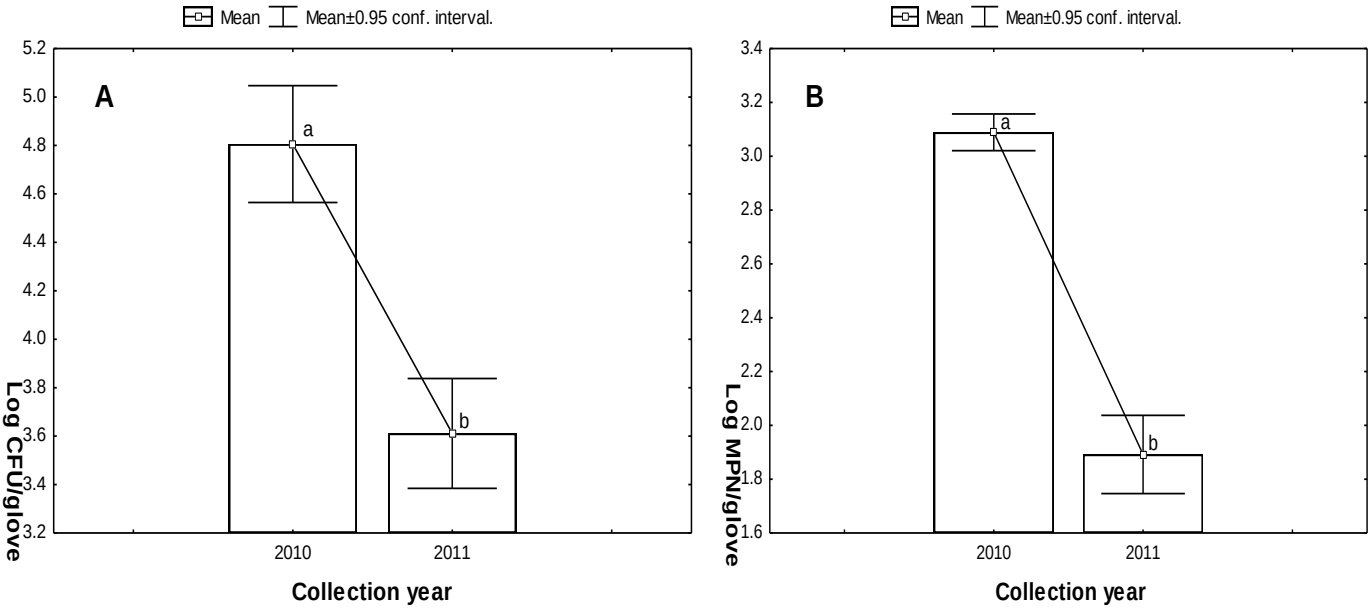

Figure 1. Comparison between the average counts of aerobic mesophiles (A) and total coliforms (B) on handlers' gloves in a fish industry collected in August 2010 and August 2011, regardless of the shift of collection. Means with different letters are significantly different ($p<0.05$).

eye on bacterial hazards and serve as warnings to maintain hygienization in the production areas.

It is crucial that all food production be organized and that the hygienization procedures, often left to second thought, be carried out effectively and uninterruptedly. The repetitive nature of the tasks and the lack of incentive favor a gradual reduction in quality, which increase the risk of pathogenic microorganism contamination. Therefore, it is important that those responsible for food companies acknowledge the value of this activity to obtain quality products from the hygienic-sanitary standpoint.

Conclusions

The results of microbiological analyses from several surfaces indicated inappropriate hygienization, especially in the first sampling period, as a consequence of the incorrect application of the Standard Operating Hygiene Procedures (SOHP). The reduction in microbial surface contamination in the second collection period was directly influenced by the implementation of GMPs in the fish processing plant. Corrective measures must still be continuously employed and the handlers' hygiene habits must be revised, particularly concerning proper rubber glove hygienization.

ACKNOWLEDGMENTS

The authors are very grateful to pro-rector of research at the Federal University of Pará for translating the original manuscript into English

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Vol. 8(5), pp. 432-436, 29 January, 2014
DOI: 10.5897/AJMR2013.6410
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Strain improvement in *Pleurotus Ostreatus* using UV light and ethyl methyl sulfonate as mutagens

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Accepted 13 January, 2014

Oyster mushroom (*Pleurotus Ostreatus*) is the choicest edible species cultivated in various regions of the world. Strain improvement studies were carried out in three strains of *P. Ostreatus* spp. Three strains of *P. Ostreatus* viz. PO-2, PO-6 and PO-7 were used for strain improvement, emphasizing on lower spore count and colour of the sporophore. It is a gymnocarpous genus of mushroom, which continuously release spores in its close vicinity causing various respiratory allergies. Their spores are highly potent allergens which can also cause exogenous allergic alveolitis. Attempts were made to produce low sporing strains of *P. Ostreatus* through mutagenesis using physical mutagen (UV light) and chemical mutagen (ethyl methyl sulfonate, EMS). Spores of three strains of *P. Ostreatus* spp. were given different treatments with UV light and EMS. Mutants exhibited appressed mycelial growth and showed slower spawn run and creamish white sporophore in PO 7(U4). A lower spore count was also observed in PO-7(E3) mutant as compared to control.

Key words: *Pleurotus*, UV light, ethyl methyl sulfonate, mutagens.

INTRODUCTION

Pleurotus spp. constitute 30% and ranks third among the cultivated mushrooms grown widely in temperate, sub-tropical and tropical regions of the world. The species of *Pleurotus* grow in the forests, attacking both cellulose and lignin components of wood (Zardazil and Kurtzman, 1982). The total world production of mushrooms is 200 thousand lakh tones in 2010. In India, mushroom production has crossed over 1,00,000 tons in 2010 (Singh et al., 2011). In the last few decades, *Pleurotus* cultivation has accelerated in India. It being a predo-minantly agriculture based country, holds a vast potential and stock of lignocellulosic waste, its adaptability to a wide range of subtropical climate temperature (20-30°C), ease of its cultivation, having good culinary and medicinal properties has attracted various farmers, entrepreneurs for its commercial production in the Indian sub continent.

The sporophores of *Pleurotus* are gymnocarpous and continuously release spores in the atmosphere causing of immunologic lung diseases like hay fever and farmer's lung disease among workers (Obatake et al., 2003) The antigens present on the walls of the spores cause the allergy. Also, during cultivation these spores settle on fruit bodies forming a velvety film after germination and thus giving an unpleasant appearance to mushroom (Ravishanker et al., 2006). The importance of fungal spores in causing air borne respiratory allergies has been well established (Hegde et al., 2002). A strict environmental control of Basidiomycetes spores is important to reduce the high risk of sensitization and possible development of various allergic diseases. A reliable spore extraction method has been devised, and a reasonable number of available patients showed positive results with

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skin test and radioallergosorbent test (RAST).

Strain improvement generally includes higher yield, better nutritional quality, colour and sporelessness. In order to overcome these constraints strain improvement has been carried out using different techniques viz. Protoplast fusion (Das and Mukheerjee, 1995), dikaryon mating (Larraya et al., 2001) and interspecific hybridization (Jaswal et al., 2013). Strain improvement in *P. Ostreatus* was first attempted using two isolates from North America which fructified well between 4-24°C and one German isolate which fructified only below 15°C (Eger et al., 1976). Mutagenesis by chemical treatment and UV irradiation has been applied for induction of sporeless mutants in *Coprinus cinerus*, *Pleurotus ostreatus* and *Pleurotus pulmonarius* (Imbernon and Labarere, 1989), AND*Agrocybe cylindracea* (Murakami, 1993). It has been reported that the mutations responsible for those sporulation defects were recessive or dominant and that most of the sporulation blockages are caused by aberration of the meiotic process or sterigma formation for sexual reproduction. Shekhar and Surjeet (2006) subjected the mycelium of *Pleurotus sajor-caju* to gamma irradiation at 10 different doses and found that with the increase in dose there was a significant decrease in the mycelial growth.

A very high variation in colour of the basidiocarp such as grey, brown or greyish black etc. is one of the limiting factors in marketing of this mushroom. Physical mutagens e.g. UV Light, gamma rays have been used for strain improvement in *P. ostreatus* (Daoping, 1997; Obatake et al., 2003; Ravishanker et al., 2006). Chemical mutagens e.g. EMS, MMS and NTG have been used to induce desirable characters like sporelessness and white colour of the basidiocarp in *P. ostreatus* (Mukherjee and Sengupta, 1986).

However, not much work has been done on the strain improvement study in *P. Ostreatus* through mutagenesis in Indian sub continent. Thus, in the present study, strain improvement in three strains of species *P. ostreatus* was aimed at achieving a lower spore count and a captivating colour of the basidiocarp through mutagenesis using physical (UV light) and chemical (ethyl methyl sulfonate) mutagens. Sporelessness is a desired trait in *P. ostreatus* causing various allergic infections in humans and causes hindrance in the commercial production of this mushroom.

MATERIALS AND METHODS

Strain improvement in *P. ostreatus* was carried out using three cultivated strains viz. PO-2, PO-6 and PO-7 for inducing white colour and sporeless fruit bodies.

UV light (physical mutagen) irradiation treatments and fruting behaviuor of *P. Ostreatus* cultures

Spore prints were made on sterile Petri plate of three selected isolates of *P. ostreatus* viz. PO-2, PO-6 and PO-7 by keeping them covered with a glass jar overnight. A dilute suspension of each spore print was made in sterile distilled water taken in test tubes and exposed to ultraviolet rays giving 8 treatments at two distances (5 and 10 cm) from source for different time of exposure (10,15, 20 and 25 min). Irradiation was given under the dark conditions to avoid the process of photoreactivation. The irradiated spore suspension was poured in sterile Petri plates containing potato dextrose agar medium and incubated at 23±2°C for 8-10 days. The mycelial bits were picked up and placed on potato dextrose agar medium slants for further studies. Mycelial growth characteristics of irradiated cultures were studied on PDA in Petri plates. From these pure cultures, spawn was prepared on wheat grains following standard procedures (Munjal, 1973). The fruiting behaviour of the irradiated cultures was studied under mushroom house conditions using pasteurized wheat straw. The fruiting pattern of irradiated cultures was compared with that of control. Observations were made on number of days for spawn run and fruiting, colour of the sporophore, sporulation. Spore count was calculated using the haemocytometer.

Chemical treatments using EMS and fruiting behavior of *P. Ostreatus* culture

Spore prints were made on sterile Petri plate of three selected isolates of *P. Ostreatus* viz. PO-2, PO-6 and PO-7 by keeping them covered with a glass jar overnight. A dilute suspension of each spore print was made in sterile distilled water taken in test tubes. The spore suspension was poured in sterile Petri plates containing potato dextrose agar medium having different concentration of mutagen ethyl methyl sulfonate (EMS) ranging from 0.001, 0.002, 0.003, 0.004 and 0.005% and incubated at 23±2°C for 8-10 days. The mycelial bits were picked up and placed on PDA slants for further studies. Mycelial growth characteristics of irradiated cultures were studied on PDA in Petri plates. From these pure cultures, spawn was prepared on wheat grains as standard procedures (Munjal, 1973). The fruiting behaviour of the EMS treated cultures was studied under mushroom house conditions using pasteurized wheat straw. The fruiting pattern of EMS treated cultures was compared with that of control. Observations were made on number of days for spawn run and fruiting, colour of the sporophore, biological efficiency and spore count was calculated using haemocytometer.

RESULTS AND DISCUSSION

Three cultivated strains viz. PO-2, PO-6 and PO-7 were used in strain improvement of species *P. ostreatus* and inducing desirable traits such as white colour of the basidiocarp and sporeless fruit bodies. *P. ostreatus* showed Clamp connections in all the three selected strains and basidiospores were oblong, ovate, hyaline in colour and size ranging from 6.5-9.5 x 3.0-4.5 µm (Figure 2).

P. ostreatus cultures irradiated with UV light

The selected isolates of *P. ostreatus* were irradiated with eight different treatments of UV light viz. T1, T2, T3, T4, T5, T6, T7 and T8. Out of which only four treatments viz

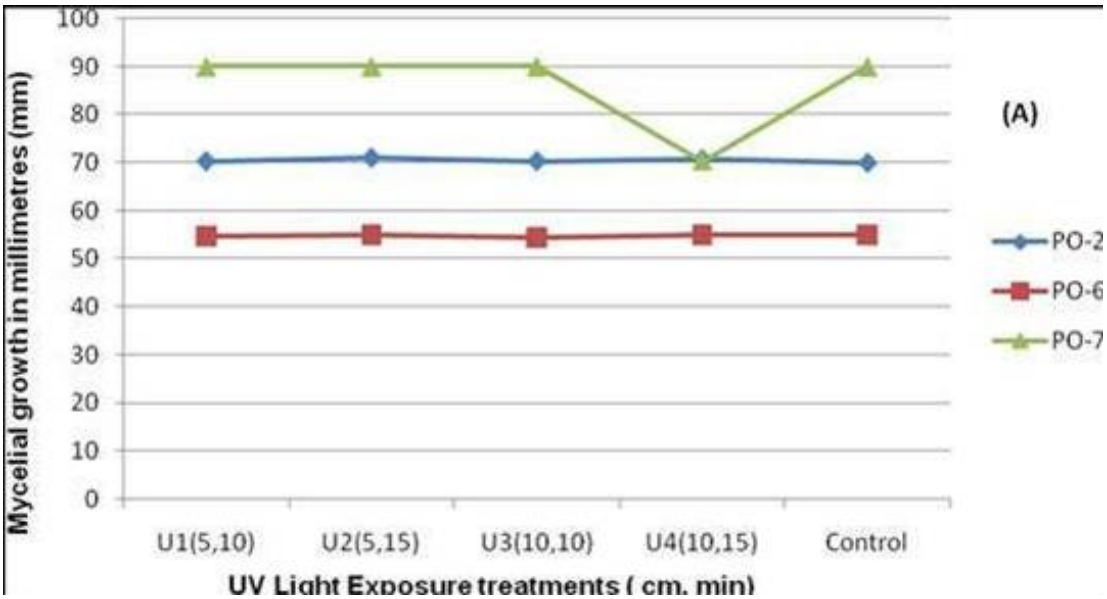

Figure 1A. Mycelial growth of three selected strains after UV light irradiation.

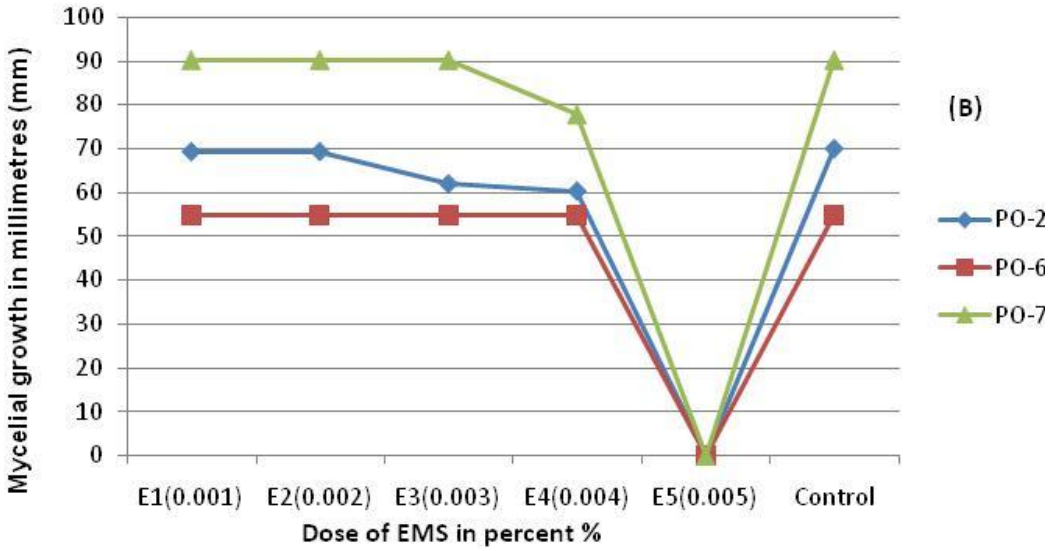

Figure 1B. Mycelial growth of three selected strains after different doses of EMS.

T1, T2, T5 and T6 supported mycelial growth in all the isolates. The irradiated isolates PO-2 and PO-6 showed no variation in mycelial growth (Figure 1A) whereas isolate PO-7(U4) showed a retarded, cottony fluffy mycelial growth 70.33 mm as compared to control which has an appressed strandy mycelial growth of 90.00 mm.

Our results are in congruence to Ravishanker et al. (2006), who also observed that with the increase in duration of exposure, the growth of mycelium retards. The mutant PO-7(U4) was further used in fruiting experiments.

P. ostreatus cultures treated with chemical mutagen (EMS)

Three selected isolates of *P. ostreatus* were treated with EMS with five treatments. Treatments with concentrations ranging from 0.001 - 0.004% showed mycelial growth whereas no mycelial growth was observed at a concentration of 0.005% (Figure 1B). Among all the treatments showing mycelial growth, a retarded mycelial growth, 62.00 and 60.33 mm was observed in PO-2(E3) and PO-2(E4), respectively (Figure 1B). A similar pattern

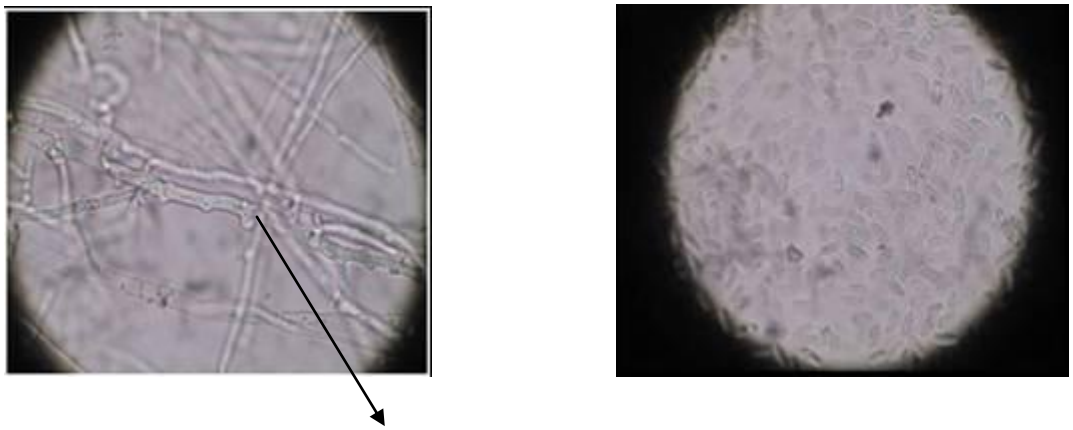

Figure 2. (A) Characteristic clamp connection and (B) basidiospores of *P. ostreatus* spp.

Figure 3. The observed change in the colour of the sporophore in mutant PO 7(U4).

of retarded mycelial growth was also observed in isolate PO-7(E4) with a 77.66 mm radial mycelial growth.

Fruiting behavior of UV and EMS treated cultures

The mutants exhibiting variations in terms of mycelial growth viz PO-7(U4), PO-2(E3), PO-2(E4) and PO-7(E4) were selected for spawn preparation. In fruiting trials of the selected mutants, data were recorded in terms of number of days for spawn run and fruiting, colour of the sporophore and sporulation. Among all the mutants, only PO-7(U4) showed creamish white colour of the sporophore in comparison with creamish brown of the control (Figure 3). However, no other significant variation was recorded in terms of biological efficiency in any of the mutants (Table 1). Lee et al. (2011) observed that basidiospores treated with chemical mutagens showed a change in primordial initiation resulting in reduced yield. Strain improvement in three isolates of *P. ostreatus* viz. PO-2, PO-6 and PO-7 using physical (UV light) and chemical mutagens (EMS) resulted in mutants showing retarded mycelial growth in PO-7(U4), PO-2(E3) and PO-2(E4). Another mutant PO-7(U4) exhibited desirable white colour of the sporophore as compared to the control. Also, a lower sporulation count was observed in PO-7(E3) while no other strains with any of the treatments showed a significant decrease in spore count.

Conclusions

Exposure of spores to UV light resulted in retarded cottony growth of mycelium 70.33 mm in mutant PO-7(U4) without causing any aberration in days for spawn run, biological efficiency and on the spore count. But mutant PO-7(U4) exhibited an attractive creamish white colour of the basidiocarp which was exposed to UV light for 15 min at 10 cm distance from the source. Similarly, spores of three strains treated at different doses of EMS,

Table 1. Fruiting behavior of selected mutants under mushroom house conditions.

Isolate	Days for spawn run (days)[a]	Colour of the sporophore	Biological efficiency[b] (%)	Effect on spore count* (cm^3/ml)
PO-2(E3)	16.66 ±1.23	Light brown	62.66 ±0.58	5.3×10^9
PO-2(E4)	16.66±1.53	Light brown	63.33 ±0.58	5.4×10^9
Control	15.55 ±0.58	Light brown	63.33 ±1.15	5.4×10^9
PO-7(U4)	14.66 ±0.58	Creamish white	62.66 ±0.58	3.7×10^9
PO-7(E3)	14.86 ±0.58	Light brown	60.33 ±0.58	2.9×10^9
Control	13.65 ±1.15	Light brown	60.66± 0.58	3.8×10^9

[a]Days for spawn run: average mean of three replications in RBD ± S.E (standard error); [b]Biological efficiency = fresh weight of mushroom / dry weight of substrate x 100% using average mean of 3 replications with RBD ± S.E (standard error); *Spore count = number of cells per ml : Number of cells/Number of squares counted x volume of a square ($1x10^{-9}$). RBD- random block design.

mutant PO-7(E4) exhibited a retarded mycelial growth of 77.66 mm at 0.004%. A lower spore count was found in mutant PO-7(U4) after conducting the fruiting trials of all the mutants. The observed results were found to be stable for more than five generations during the stipulated time of the research duration. Although, if time permits we would test the stability of the improved character for more than ten generations.

ACKNOWLEDGEMENTS

Authors are highly thankful to National Horticulture Board, India for providing funds under the mushroom production and training project. We are also grateful to CSK HP KV University for timely help and cooperation.

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academicJournals

Vol. 8(5), pp. 441-445, 29 January, 2014
DOI: 10.5897/AJMR2013.6359
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and antibiogram of pneumonic pasteurellosis causing microbes from nasopharynx of transport stressed Nigerian goats

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Accepted 13 January, 2013

This investigation reports the isolation and antibiogram of pneumonic pasteurellosis causing microbes, *Mannheimia haemolytica* (MH) and *Pasturella multocida* (PM) from the nasopharynx of transport stressed Nigerian goats. Nasal swabs were taken from 63 that were transport stressed (group A), 21 goats that were transport stressed followed by rest for two weeks (group B) and 21 resident goats which were not transported (group C). The characterisation, identification of the isolates was carried out using standard methods while the antibiotics sensitivity test was by disc diffusion technique. The rate of isolation and load of MH and PM from the nasal cavity was significantly higher in transport-stressed goats while there was no significance difference between groups B and C. The duration of journey did not significantly affect the isolation rate of the two organisms except the bacterial load of MH. Most isolates were susceptible to the quinolones (cefuroxime, ciprofloxacin and ofloxacin) and resistant to gentamycin, augmentin, nitrofurantoin, ceflazidime and ampicillin. Efforts should be geared towards improving state of Nigerian roads, animal transportation and the treatment plan that is commonly used for stabilising transport-stressed animals especially with the use of rest and appropriate antibiotics prophylaxis.

Key words: Goats, transport-stress, *Mannheimia hemolytica*, *Pasteurella multocida*, Nigeria.

INTRODUCTION

The management of goats in most parts of Africa is often extensive and it's primarily in the hands of women and children. They commonly serve as source of income and meat to the rural poor (Diallo, 2006). Goats thrive in most ecological zones but often than not, they predominantly domiciled in the arid and semi arid areas of Nigeria where there are vast expanse of land to support their production. The transportation of such animals from this area to other parts had become an age long, inevitable husbandry practice (Fazio and Ferlazzo, 2003; Minka and Ayo, 2007) especially in Nigeria where most of the food animals are often transported from the dry north to the humid south by road, either to be slaughtered or intensively reared. However there is no concrete information or policy on the transportation of animals in Nigeria, compared to the European Union.

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Transportation of animals as defined by the European directive, (European Union (EU) 2002) involves road transportation of live animals, in which the process of handling, loading and unloading had been regarded as stressful period than the journey itself (Minka and Ayo, 2007). During this period of transportation, the health and productivity of animals are significantly affected due to the deplorable state of major roads in Nigeria (Meludu, 2008) and the long distance in which the animals are subjected to.

The need to understand the behaviour, the physiological and pathological derangements associated with the transport of animal under these deplorable conditions when moved from one zone to the other cannot be overemphasised. This understanding will help in ensuring enforcement of code of practice/legislative control on animal welfare (Grandin, 1989) which will go a long way to ensure the safety of the transported animals and their handlers.

Numerous investigations abound on the welfare of livestock during road transportation and the effects on behavioural changes and physiological parameters (Kannan et al., 2000; Ayo et al., 2002; 2006, Odore et al., 2004; Minka and Ayo, 2007) but very little information are on the pathological derangements (Jasni et al., 1991; Emikpe et al., 2013) albeit the bacteriological changes.

In Nigeria, majority of the slaughtered and breeding goats transported down south by road (Minka and Ayo, 2007) are often predisposed to respiratory diseases which include peste des petits ruminants (PPR) and *Mannheimiosis* (*Pasteurellosis*, Shipping fever). The two diseases had been observed to co-exist in nature and the combined infection had been reported to be fatal (Emikpe et al., 2010).

The bacteria usually associated with caprine pneumonia especially pneumonic pasteurellosis are *Mannheimia haemolytica* (MH) and *Pasteurella. multocida* (PM) (Brogden et al., 1998, Emikpe et al., 2013). They are normal flora of the respiratory tract of goats (Emikpe et al., 2009) which can be pathogenic when the animal is subjected to stressful conditions. In Nigeria, with the deteriorated state of roads, transporting vehicles and the fact that animals are transported over more than 1 500 to 2 000 km which could span days, the possibility of being predisposed to respiratory infection is high.

With the investigations on the bacterial flora of the respiratory tract of Nigerian goats being on the pneumonic lungs (Tijjani et al., 2012a,b) and apparently normal nasal passage (Emikpe et al., 2009), it is expedient to understand the effect of transport stress on the nasal bacterial flora especially pneumonic pasteurellosis causing microbes, *Mannheimia haemolytica* (MH) and *Pasturella multocida* (PM). The aim of the present study was to investigate the effect of transport, as stress factor, on the isolation of pneumonic pasteurellosis causing microbes from nasopharynx of goats as well as the possible implications and subsequently method of control.

MATERIALS AND METHODS

Study area

Ibadan city is situated at latitude 07°20´ North and longitude 03°50´ East. Ibadan is one of the largest urban cities in Nigeria and West Africa with over one million residents and thousands of daily migrants. Bodija small ruminant market is a spacious market constructed by the Oyo State government. Sheep and goats from different parts of Nigeria are brought to the market and subsequently conveyed to different abattoirs, farms and households throughout the state.

Animals

A total of one hundred and five Nigerian goats were purposely selected based on the criteria used for this investigation which include transportation, duration of transport and age of the goats. Nasal swabs were taken from twenty one goats per location used (Niger, Kaduna, Sokoto and Miaduguri) which are state capital cities where goats are often transported to the study area. The goats were divided into three groups, group A: 63 goats with transport stress; group B: 21 goats with transport stress followed by rest for two weeks and; group C: 21 resident goats without any stress were used as control. The goats enrolled in this study were between two to three years old.

The distance of transportation ranged between from 350 to 1 800 km to the point of samples collection (Figure 1). The goats were fed with chopped dry grass and groundnut chaffs while water was supplied ad libitum before the commencement of the journey however; the goats were often not fed or given water in the course of the journey which could take two to four days.

Sample collection

Nasal swabs were collected from goats after arrival (group A), those that have rested for two weeks after arrival (group B) and from resident goats within the area, not stressed (group C, Control). Nasal samples were collected aseptically in all the groups by inserting sterile cotton tipped applicator sticks or swab into the nasal passage after proper cleaning and disinfection of the external nares. Each nasal swab on swab stick was carefully cut into a well labelled bottle containing 2 ml brain heart infusion broth. The swabs were transported in a deep fridge cold box to the laboratory for bacterial isolation.

Bacteriological examination

The characterization and identification of the bacterial isolates were carried out using standard methods as previously described by Quinn et al. (1994). Each nasal swab was removed from the bottle and streaked over the plates containing blood agar – base supplemented with 7% sheep blood and MacConkey agar. The streaking was further spread with inoculating loop to aid colony isolation. The plates were labelled and incubated aerobically at 37°C for 48 h (Carter, 1984). After taking note of cultural growth characteristics, positive cultures were subjected to Gram's staining properties and cellular morphology under 1000x objective of light microscope. Mixed colonies and Gram-negative bacteria were sub cultured on both blood and McConkey agars and incubated

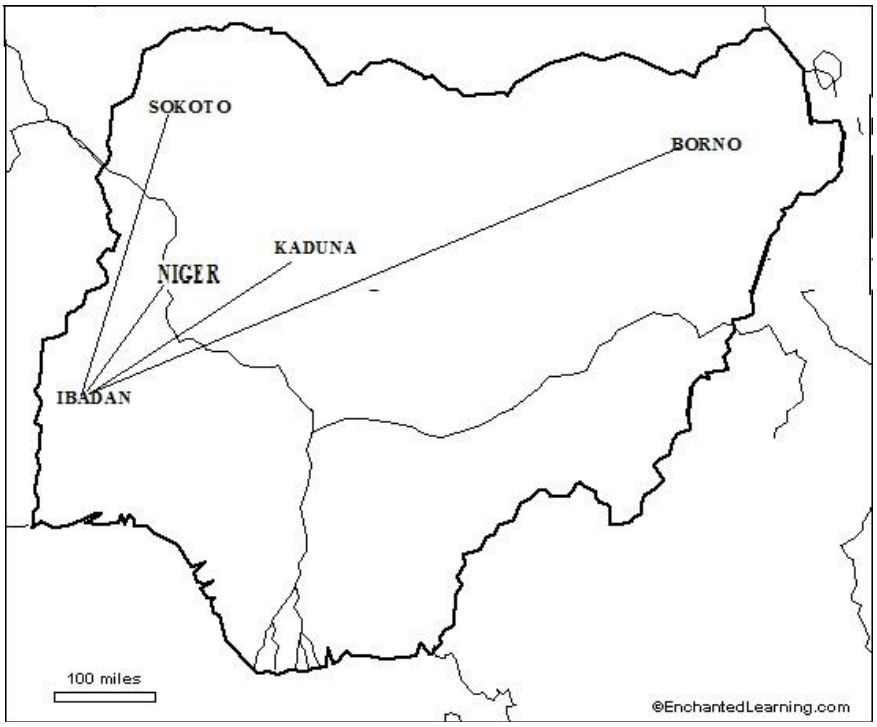

Figure 1. The areas where goats are often transported to the study area.

Table 1. Effect of transport stress on the isolation rate and nasal *Mannheimia haemolytica* and *Pasteurella multocida* load.

Grouping	*Mannheimia haemolytica* isolation rate (%)	Load × 10^8	*Pasteurella multocida* Isolation rate (%)	Load × 10^8
Transported	70	70.5 ± 5.5	95	47.2 ± 4.5
Two weeks after	70	11.3* ± 2.3	100	21.5* ± 3.4
Control (not transported)	80	6.6* ± 1.5	90	15.3* ± 2.5

*Not significantly different (p>0.05).

aerobically for further 24 h. Pure culture of single colony type, from both blood and McConkey agars were transferred onto nutrient agar- slants for a series of biochemical tests including catalase, oxidase and fermentative/oxidative tests for final identification following standard procedures (Quinn et al., 1994). All the isolates of *P. multocida* were catalase and indole positive and did not grow on MacConkey agar while those of *Mannheimia hemolytica* grew on MacConkey and did not produce indole (Tefera and Smola, 2002). Further identification using API test kit was not attempted.

For antibiotic sensitivity test, newer and some common anti-biotics were used. The antibiotics include cephalosporins (cefuroxime, ciprofloxacin and ofloxacin), gentamycin, augmentin, nitrofurantoin and ampicillin. The disc diffusion technique was employed and inhibition observed as clear zones around the antibiotics. Inhibition zones were measured using meter rule and measurement greater than 0.5 cm was regarded as susceptibility (Emikpe et al., 2009).

Statistical analysis

Descriptive statistics was used to summarise the data generated from the study. The effect of transport and duration were evaluated using one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences 2006, version 15.0). Duncan's multiple range tests were used to compare differences among individual means.

RESULTS

The frequency and the load of MH and PM isolation were varied within the groups as shown in Table 1. The isolation and load of MH and PM from the nasal cavity were significantly *(P<0.05)* higher in transport stressed goats than goats rested for two weeks or not subjected to transportation stress.

The effect of duration of journey on the isolation rate and nasal bacterial load were shown in Table 2. The duration of journey did not significantly *(P < 0.05)* affect the isolation rates of MH and PM, while the MH load was significantly affected by the distance of journey.

Table 2. Effect of the distance of journey on the isolation rate of nasal *Mannheimia haemolytica* and *Pasteurella multocida* load.

Duration of journey	MH isolation rate (%)	Load × 10^8	PM isolation rates (%)	Load × 10^8
2 days	80	$52.0^{(a)} \pm 4.3$	100	$47.2^{(a)} \pm 4.5$
3 days	50	$26.7^{(a)} \pm 3.5$	83.3	$21.5^{(b)} \pm 3.3$
4 days	70	108.0 ± 7.4	80.0	$56.0^{(a)} \pm 4.2$
Control (not transported)	0	6.6 ± 2.1	90.0	$15.3^{(b)} \pm 2.4$

*Same alphabet in the same group are not significantly different $p>0.05$.

Table 3. Anti microbial sensitivity pattern of the isolation rate of nasal *Mannheimia haemolytica* and *Pasteurella multocida*.

Bacteria	AMP	CAZ	CRX	GEM	CPR	OFL	AUG	NIT
Mannhemia haemolytica	R	R	S	S	S	S	R	R
Pasteurella multocida	R	R	PS	S	S	S	R	S

R: resistant, S: sensitive, PS: partially sensitive, AMP: ampicillin 25 µg, CAZ, ceflazidime 30 µg, CRX cefuroxime 30 µg, GEN gentamicin 30 µg, CPR ciprofloxacin 10 µg, OFL, ofloxacin 5 µg, AUGaugmentin 30 µg, NIT nitrofurantoin 300 µg.

The antibiotics resistance pattern showed that the isolates were resistant to ampicillin, gentamycin and augmentin, but susceptible to cefuroxime, ciprofloxacin and ofloxacin (Table 3).

DISCUSSION

The significant higher percentage of isolation and load value of MH and PM in the nasal cavity of transport stressed goats in this study suggest their potential risk in the establishment of pneumopathy in stressed animals (Brogden et al., 1998; Emikpe et al., 2013). *Mannheimia haemolytica* and *Pasteurella multocida* were isolated immediately after the arrival of the transported goats which suggested that transportation could enhance the isolation rate of the organisms from the nasal cavity of transported goats. Duration of journey might increased the chance of isolation as opined by some workers (Zamri-Saad et al.,1989) as time is required for active proliferation and persistence of the bacteria in the nasal cavity. The mechanism of the persistence and proliferation is that stress factors with or without viral infection suppress the mucociliary clearance (Brogden et al., 1998) and an abrupt shift from commensals to pathogenic (Gonzalez and Maheswaran, 1993) form which may account for their involvement in most pneumonia associated with stress.

Resting allowance of two weeks after arrival could alleviate the transport-stress and reduce the proliferation of *Mannheimia haemolytica* and *Pasteurella multocida* in the nasopharnyx with time (Pass and Thompson, 1971). This observed marked reduction in bacterial load and isolation rate possibly due to acclimatization may account for the usual two weeks stabilization of animals after stressful farm operations and in quarantine procedure (Zamri-Saad et al., 1989).

In this study, we observed no significant *(P<0.05)* correlation between PM load or isolation rate and the distance of journey while in MH, there was a significant *(P<0.05)* correlation between bacterial load and the distance of journey, this observation further lend credence to the fact that MH is more associated with transport stress than PM in Nigeria unlike the reverse in Malaysia (Emikpe et al., 2013). The possible effect of distance and the period of resting phases in the course of the journey on the bacterial load may need to be further investigated.

In this study, the drug resistance pattern was similar for the two bacteria with isolates being susceptible to the cephalosporins (cefuroxime, ciprofloxacin and ofloxacin) while most were resistant to gentamycin, augmentin, nitrofurantoin, ceflazidime and ampicillin. The antibiotic sensitivity pattern observed further revealed that the bacteria are resistant to expensive and newer antibiotics and were resistant to common antibiotics which are easily bought off the shelf without appropriate prescription from practicing veterinarians and are often prone to adulteration.

Conclusion

Efforts should be geared towards improving the treatment plan that is commonly used for stabilising transport-stressed animals especially with appropriate antibiotics prophylaxis. The prophylaxis may possibly involve vaccination strategies which may help in the control of bacte-

rial pneumo-pathy associated with road transport in small ruminants. Effort should also be geared toward improving the state of the roads in Nigeria to reduce the stress associated with road transportation.

ACKNOWLEDGEMENTS

The author is grateful to Professor M. Zamri-Saad and Dr. Md Sabri Yussof, Department of Pathology and Microbiology, Faculty of Perubatan Veterinar, University of Putra, Malaysia for their assistance.

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Vol. 8(5), pp. 446-451, 29 January, 2014
DOI: 10.5897/AJMR2012.2457
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibacterial activity of the curcumin derivative FM0817 against *Neisseria gonorrhoeae*

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Accepted 16 July, 2013

The objectives were to analyze the anti-bacterial activity of the curcumin derivative FM0817 and its mechanism of action. We first tested 40 strains of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) for their susceptibilities to antibiotics by the Kirby-Bauer and agar dilution methods. Total DNA was extracted by the boiling method, and 22 strains of *N. gonorrhoeae* were randomly selected and used as templates for polymerase chain reaction (PCR) amplification of the antibiotic-resistance genes: *TEM*, *tetM*, *erm,* and *mefA*. The filter disc diffusion method was used to determine whether FM0817 had anti-bacterial activity. Eight strains, covering four drug-resistant genotypes/phenotypes and five different types of resistance, were selected. The susceptibility tests confirmed multi-drug resistance in *N. gonorrhoeae*. The percentage of strains possessing *TEM* and *tetM* was 77.2 and 77.2%, respectively. The drug-resistance genes, *erm* and *mefA*, were not detected in any of the strains studied. FM0817 exhibited potent anti-bacterial activity *in vitro*, but the relationship between FM0817 activity and multi-drug resistance and the resistance genes of *N. gonorrhoeae* remains unclear, therefore, it requires further investigation.

Key words: Curcumin, anti-bacterial activity, *Neisseria gonorrhoeae*, resistance genes.

INTRODUCTION

Neisseria gonorrhoeae is a Gram-negative pathogenic bacterium responsible for an array of diseases ranging from urethritis to disseminated gonococcal infection. The incidence rate of gonorrhea, the disease caused by *N. gonorrhoeae*, is currently high due to the rapid spread of this pathogen. The United States Centers for Disease Control stated that the penicillin and tetracycline were not the first line of drugs because of the high drug-resistance rates (Gonzalez et al., 2009). It is time to sound the alarm. During the past three years, the wily gonococcus has become less susceptible to our last line of antimicrobial defense, threatening our ability to cure gonorrhea and prevent severe sequelae, and it was needful to find the new drugs (Bolan et al., 2012).

Curcumin is a monomeric phenolic compund, belonging to the ginger family (Zingiberaceae), and it has widespread pharmacological activities, including anti-tumour oxidation-resistance, anti-sudden change activity, anti-inflammatory, and anti-blood platelet accumulation activity (WG, 1997). In recent years, curcumin pharmacological studies focusing, in particular, on the inhibition of tumour cell proliferation and on the induction of apoptosis have gained attention, along with the *in vitro* anti-bacterial action of curcumin (Na et al., 2011). The water-solubility of ginger flavine is poor, but hydrolysis can be carried out in peroxide solution. These factors have limited its biological application and have caused an industrial production bottleneck. Studies have been per-

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Table 1. Primers used to amplify *Neisseria gonorrhoeae* drug-resistant genes.

Drug-resistance gene	Primer sequence	Product size (bp)
TEM gene exon	P1: 5′-ATAAAATTCTTGAAAACGAAA-3′ P2: 5′-GACAGTTACCAATGCTTAATCA-3′	1074
TEM gene within exon	P3: 5′-AGGAAGAGTATGATTCAACA-3′ P4: 5′-CTCGTCGTTTGGTATGGC-3′	535
tetM	P1: 5′-GTGGACGAACTTTACCGAA-3′ P2: 5′-GCTTTGTATCTCCAAGAACAC-3′	501
erm	P1: 5′-GGATACGGTTTAGATATTGGG-3′ P2: 5′-TTGAAGGACAATGGAACCTCC-3′	295
mefA	P1: 5′-ACTATCATTAATCACTAGTGC-3′ P2: 5′-TTCTTCTGGTACTAAAAGTGG-3′	346

formed to modify the ginger flavine structure by retaining the ginger flavine aromatic ring structure and replacing the single-carbonyl piperidine hydrochloride 42, but not the saturated B2 two alkones, resulting in the synthesis of a new ginger flavine derivative, designated FM0817 (YX, 2000, Shoba et al., 1998).

A study comparing FM0817 and ginger flavine has been reported (NW Zhang et al., 2009). The aim of this study was to determine the anti-bacterial activity of the curcumin derivative FM0817 on *N. gonorrhoeae*, along with its mechanism of action.

MATERIALS AND METHODS

Specimen origin and gonococcus isolation

Clinical strains of *N. gonorrhoeae* were isolated from specimens submitted to the Guangdong Province Zhongshan People's Hospital from January 2009 to August 2010. Clinical specimens received by the hospital were examined according to the "Nation Clinical Checked Operation Regulations". *N. gonorrhoeae* isolated from specimens were cultured, and were identified by API NH.

Drug sensitive analyses

The antibiotic sensitivity of forty gonococcal isolates were tested by the National Center for Clinical Laboratory (China) using the Kirby-Bauer. The results were interpreted according to the standard reference, CLSI (2008). The gonococcus (Zopf) Trevisan standard strain, ATCC 49226, was used as a positive control.

Extraction of bacterial DNA

Bacterial DNA was extracted according to the previously published boiling method (Tanaka et al., 1998).

The sequence of drug-resistant genes primers

Previously reported primers were used in this study (Li et al., 2005), as shown in Table 1. Primers were synthesized by Boya Biological Corporation.

Polymerase chain reaction (PCR) amplification of drug-resistance genes

PCR reactions (total volume of 25 µl) comprised TaqE reaction buffer, 12.5 µl of dNTPs, 1 µl each of forward (F) and reverse (R) primers, and 1 µl of DNA template. The reaction conditions involved DNA amplification by 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, with a final extension step of 72°C for 7 min. After 2% agarose gel electrophoresis, the DNA bands were visualized and recorded via a computer image analyzer. The *TEM* gene from *Escherichia coli* and the *tetM* gene from *Staphylococcus aureus* were used as positive control genes.

Anti-bacterial activity of the curcumin derivative FM0817

The disc diffusion method was used to determine the anti-bacterial activity of FM0817. Among the clinical *N. gonorrhoeae* isolates obtained, 8 strains, covering four drug-resistant genotypes and five drug-resistance phenotypes, were selected for this experiment, as shown in Table 2.

The inculum was prepared in normal saline to turbidity equivalent to a 0.5 McFarland standard (ca. 1.5 x 10^8 colony-forming units (CFU)/ml) and was swabbed on Mueller-Hinton agar. Every strain was inoculated in three Mueller-Hinton agar, respectively, Three kind of discs were used in each Mueller-Hinton agar, the bank, curcumin and curcumin derivatives FM0817 filter disc. The experiment was repeated three times.

Qualitative filters made of 6 mm disks, soaked in sterile water, were applied, along with FM0817 curcumin, to the centre of three Petri dishes. The dishes were then incubated in a 5-10% thermostatic CO_2 cultivation cabinet, for 24 h at 20-35°C. After removing the disk, bacteriostatic measurements were made and the average values were recorded.

Statistical analysis

The inhibition zones among different drug-resistance gene mutations were analyzed using the SPSS software.

RESULTS

Drug sensitivity of the *N. gonorrhoeae* strains

All 40 of the *N. gonorrhoeae* strains tested were resistant to penicillin. All strains were suscesptible to spectinomycin and cefuroxime axetil. The levofloxacin, ciprofloxacin, and tetracycline-resistance rates of the strains were 95.0, 92.5, and 62.5%, respectively, as shown in Table 3. *N. gonorrhoeae* strains, representing eight antibiotic-resistance phenotypes, were divided into five

Table 2. Kirby-Bauer anti-microbial susceptibility testing of *N. gonorrhoeae* strains isolated from January 2009 to August 2010 in Zhongshan district (*n* = 29).

Antibiotic	Resistance rate (%R)	Mid-sensitive rate (%I)	Sensitive rate (%S)
Cefoxitin	10 (4/40)	2.5 (1/40)	87.5 (35/40)
Cefuroxime axetil	0 (0/40)	7.5 (3/40)	92.5 (37/40)
Ciprofloxacin	92.5 (37/40)	7.5 (3/40)	0 (0/29)
Ceftriaxone	5.0 (2/40)	2.5 (2/40)	92.5 (37/40)
Ofloxacin	95.0 (38/40)	5.0 (2/40)	0 (0/40)
Penicillin	75.0 (30/40)	25.0 (10/29)	0 (0/40)
Spectinomycin	0 (0/40)	2.5 (1/40)	97.5 (39/40)
Tetracycline	62.5 (25/40)	25.0 (10/29)	12.5 (5/40)

Table 3. Resistance to eight antibiotic species among the *Neisseria gonorrhoeae* strains isolated from January 2009 to August 2010 in Zhongshan district (n=40).

Resistant to antibiotic species[&] (n=8)	Number of strains (*n*=40)	Rate of resistance (%)
1	1	2.5
2	5	12.5
3	11	27.5
4	19	47.5
5	4	10.0
Total	40	100

[&] the number of antibiotics to which a given strain showed resistance

groups based on drug-resistance antibiotic species, as shown in Table 3.

The prevalence of drug-resistance genes among *N. gonorrhoeae* strains

The four drug-resistance genes of twenty-two strains were detected in this study (Figures 1-3). The gene-mutation rates of *TEM* and *tetM* were both 77.2%, and the *erm* and *mefA* genes were not detected. These data showed the enzymes were responsible to the penicillin resistance.

The drug-resistance rate of tetracycline was high (62.5%), but there was no enzyme detected. There were other drug-resistance mechanisms. Twenty-two (22) *N. gonorrhoeae* strains tested were divided to four mutation types (Table 4). Thirty (30) strains were detected both *TEM* and *tetM* genes (59.1%). Four strains were detected *TEM* gene (18.1%). Four strains were detected *tetM* gene (18.1%). There was one strains what were not drug-resistance genes detected (4.7%).

Anti-bacterial activity of the curcumin derivative FM0817

The curcumin derivative, FM0817, was tested *in vitro* for its activity against *N. gonorrhoeae*. For eight of the *N. gonorrhoeae* strains, bacteriostatic annulus diameters of between 33-37 mm were observed, but the blank disc and the curcumin disc had no inhibition zone. It indicated that the FM0817 had the activity of inhibiting *N. gonorrhoeae*. However, no significant differences were detected about the inhibition zones among different drug-resistance gene mutations. Taken together these data suggest there was no relation between the inhibition activity and the mutation kinds.

DISCUSSION

N. gonorrhoeae has always readily developed resistance to antimicrobial agents: it became resistant to sulfanilamide in the 1940s, penicillins and tetracyclines in the 1980s, and fluoroquinolones by 2007 (WHO report, 2007). When the prevalence of antimicrobial resistance in the gonococcal isolate surveillance project (GISP) exceeds 5%, national treatment recommendations are changed to focus on other effective drugs.

However, the treatment options recommended by the Centers for Disease Control and Prevention (CDC) are now limited to third-generation cephalosporins. But susceptibility to cephalosporins decreasing rapidly (Katz et al., 2012). In the search for new compounds with biological activity curcumin and derivatives therefore

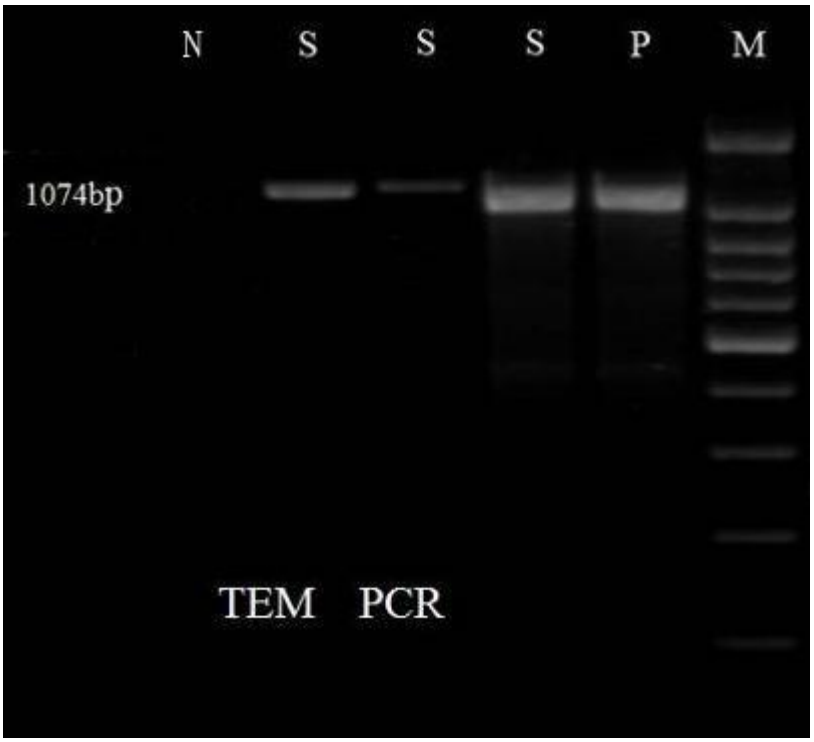

Figure 1. PCR amplification of the TEM gene. M, DNA marker (DL1500); S, sample; P, positive control (contains TEM-1 gene of E. coli); N, negative control.

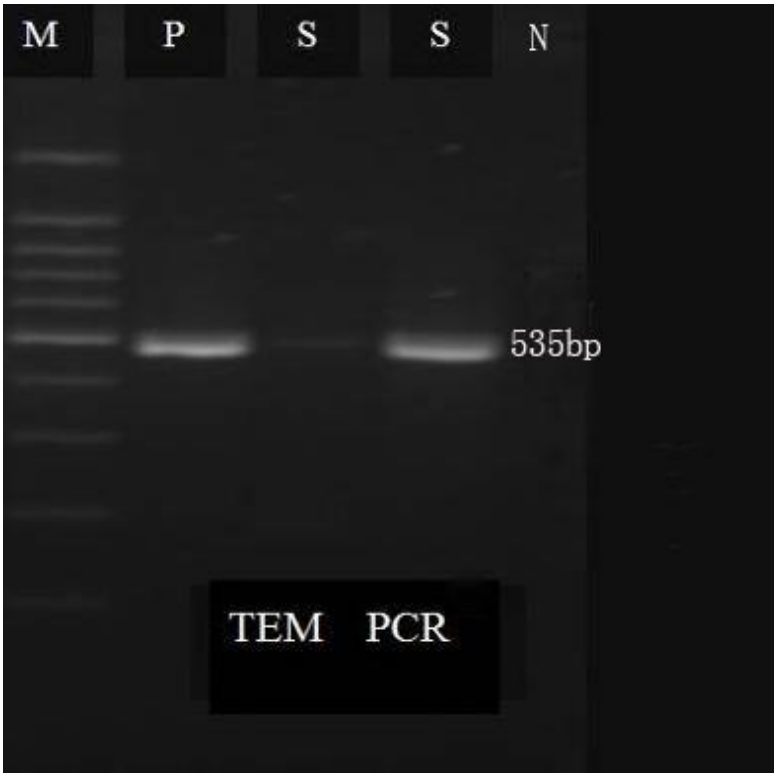

Figure 2. PCR amplification of the *TEM* gene. M, DNA marker (DL1500); S, sample; P, positive control (contains *TEM-1* gene of *E. coli*); N, negative control.

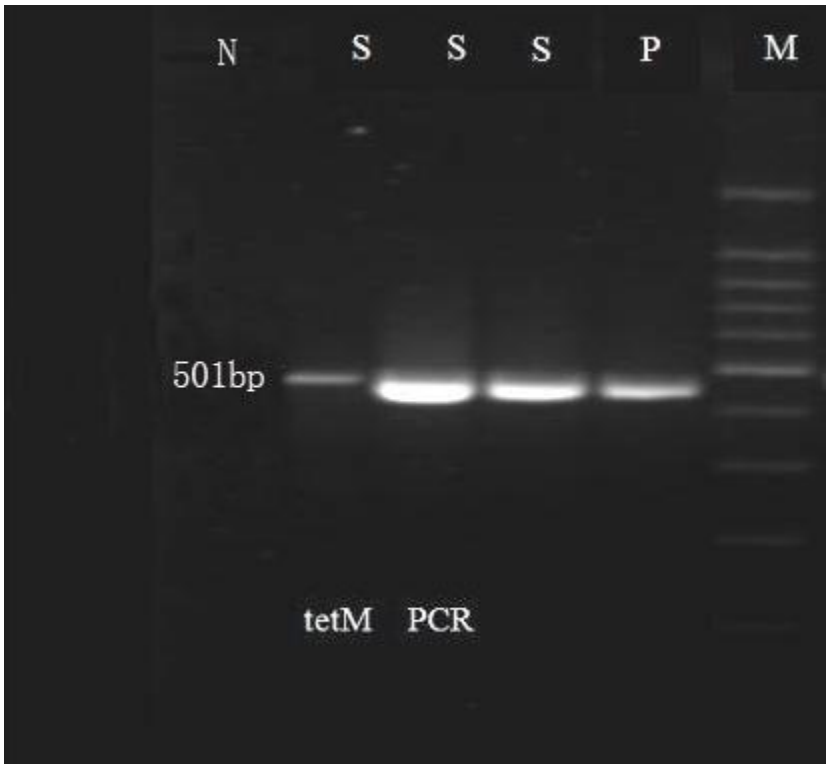

Figure 3. PCR amplification of the *tetM* gene. M, DNA marker (DL1500); S, sample; P, positive control (contains *tetM* gene of *S. aureus*); N, negative control.

Table 4. Prevalence of drug-resistant genes in 22 strains of *Neisseria gonorrhoeae*.

Genotype	Number of strains	Percentage
TEM- tetM-	1	4.7
TEM+ tetM+	13	59.1
TEM- tetM+	4	18.1
TEM+ tetM-	4	18.1

All strains were negative for the *mefA* and *erm* genes

have attracted the interest of scientists.

To explore the activity of curcumin on *E. coli* in a sepsis mice protection model, Hou et al. (2008) randomly divided Kunming mice into control, model and curcumin-treated groups. The results were proposed that curcumin could reduce TNF-α, NO, and oxygen free radicals in mice with bacterial sepsis, reducing the viscera pathological damage.

There were several researchers who investigated the anti-microbial activity of curcumin and its derivatives *in vitro* (Na et al., 2011; Zhong et al., 2008). The results show that curcumin may be an alternative antimicrobial agent against fetal bacterial infections.

Based on the molecular structure of curcumin, containing two carbonyl groups, they used aniline, 4-methyl aniline, and benzene nitrofication as hydrazines, and 2,4-difloronitrohenzene hydrazine curcumin as the raw material to synthesize four kinds of Schiff base curcumin stability derivatives. The analysis of *in vitro* antimicrobial activity revealed that four curcumin derivatives, including Schiff bases, had good antibacterial activity (Zhang et al., 2009).

In the present study, 40 strains of penicillin-resistant *N. gonorrhoeae* were analyzed. Cefuroxime axetil and spectinomycin-resistant strains that have high drug-resistance to ofloxacin, ciprofloxacin, and tetracycline are not known to be prevalent in China. The detection rates for penicillin and tetracycline-resistance genes recorded in this study were significantly higher than those reported by Li et al. (2012); likely due to the widespread application of antibiotics and the rapid spread of drug-resistance genes.

The erythromycin-resistance-related methylation enzyme, encoded by the *erm* gene and the *mefA* gene, were not detected in our study. Analysis of the prevalence of drug resistance among *N. gonorrhoeae* strains

Table 5. FM0817 *in vitro* experiments with selected strains of *N. gonorrhoeae.*

Number of strains[&]	Genotype	Diameters of bacteriostatic annulus (mm)[#]		
		The blank	Curcumin	FM0817
22	*TEM+ tetM-*	6	6	35
9	*TEM- tetM+*	6	6	34
16	*TEM- tetM-*	6	6	35
18	*TEM+ tetM+*	6	6	36
20	*TEM- tetM+*	6	6	36
5	*TEM+ tetM+*	6	6	37
7	*TEM+ tetM-*	6	6	36
17	*TEM+ tetM+*	6	6	34

[&]Identification number of what was given; [#]diameter of 6.0 mm is included in the inhibition zone disc ammeter.

throughout the world is complicated by regional genotype/phenotype differences. Strengthening resistance monitoring, the careful management of antimicrobial usage and further research into *N. gonorrhoeae* drug-resistance mechanisms are indispensable for controlling drug resistance in this organism. This study reveals *N. gonorrhoeae* resistance genotypes/phenotypes and provides insight into a new antimicrobial agent, which could potentially be used in the control of *N. gonorrhoeae* infection.

N. gonorrhoeae infection induces inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 expression, and curcumin can inhibit IKKalpha, IKKbeta and NF-κB, showing that its powerful induced kinase from NF-κB mediates cytokine release and induces innate immune responses (Wessler et al., 2005). Except in the case of *N. gonorrhoeae* inflam-mation, the study also showed that curcumin at a late stage of infection may fully inhibit bacteria adhered to cells, a huge potential side effect of non-toxic antimi-crobial drugs.

In our experiments with curcumin using eight strains of *N. gonorrhoeae*, bacteriostatic annulus diameters of 6 mm were recorded (the diameter of the disc), indicating no bacteriostatic activity. However, this result was different to the result obtained from the cells (Wessler et al., 2005). On testing FM0817 derivatives of curcumin against eight strains of *N. gonorrhoeae*, the bacteriostatic annulus diameters showed no obvious differences in size (33-37 mm on average). The mechanism of action of FM0817 and the *N. gonorrhoeae* drug-resistance genotypes/phenotypes may not be related, and, furthermore, our analysis of drug-resistance strains and the genotypes/phenotypes of drug resistance may not be comprehensive. Therefore, further analysis of different resistance genotypes and drug-resistance strains of different phenotypes with respect to FM0817 antimicrobial susceptibility is required. However, the FM0817 derivative of curcumin showed larger bacteriostatic annulus diameters (average 35 mm) with eight strains of *N. gonorrhoeae*, showing antibacterial activity *in vitro.* Further research is now required to fully elucidate the antimicrobial properties and potential applications of the FM0817 curcumin derivative.

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Vol. 8(5), pp. 452-457, 29 January, 2014
DOI: 10.5897/AJMR2013.5859
ISSN 1996-0808 ©2014 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Sandwich enzyme-linked immunosorbent assay (ELISA) method for reliable detection *Burkholderia pseudomallei* in soil samples

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Accepted 13 January, 2014

***Bulkholderia pseudomallei*, the pathogeny of melioidosis, is naturally occurring in soil and water in epidemic regions. The environmental distribution of *B. pseudomallei* is still unclear, as indeed is the global distribution of *B. pseudomallei.* To detect *B. pseudomallei* in epidemic regions, a double antibodies sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed to detect *B. pseudomallei* in soil cultures directly using two monoclonal antibodies (Mab) specific to recombinant truncated flagellin of *B. pseudomallei*. The method was evaluated by detecting the 154 soil samples collected from the Guangxi region in China where the endemic of melioidosis is. The results indicated that the sandwich ELISA method is not only faster than culture method which is the gold standard for *B. pseudomallei* detection in soil, but also a more sensitive method. It provides a new tool to detect *B. pseudomallei* in soil samples and is applicable for large screening studies to assess the distribution of *B. pseudomallei* in the endemic of melioidosis.**

Key words: *Burkholderia pseudomallei*, melioidosis, enzyme-linked immunosorbent assay (ELISA), soil sample.

INTRODUCTION

Melioidosis, an infectious disease caused by the Gram-negative organism *Bulkholderia pseudomallei,* is now recognized as an important public health problem in South-east Asia and tropical Northern Australia (Cheng and Currie, 2005). *B. pseudomallei* is a soil-dwelling saprophyte, naturally occurring in rice-farming fields, rubber plantations, cleared fields, cultivated and irrigated agricultural sites and water in endemic regions (Raja et al., 2005). Despite the detection of *B. pseudomallei* in various water and soil samples in Southern-east China (Su et al., 2007; Yang, 2000), the environmental distribution of *B. pseudomallei* is still unclear, as indeed is the global distribution of *B. pseudomallei.*

The gold standard for *B. pseudomallei* detection in soil is culture, which is time-consuming and takes up to weeks for final results (Novak et al., 2006). Molecular detection techniques such as direct soil DNA isolation and PCR have been successfully applied to detect *B. pseudomallei* (Kaestli et al., 2007). Also monoclonal antibody were used to rapid identify *B. pseudomallei* in blood cultures (Pongsunk et al., 1999; Steinmetz et al., 1996). But there are no methods which can rapidly detect *B. pseudomallei* directly in soil cultures.

In this study, we tried to shorten the detection time by ELISA method using two monoclonal antibodies (Mab) specific to recombinant truncated flagellin of *B. pseudomallei* to detection the bacteria in soil cultures. By this method, detection time of *B. pseudomallei* in soil samples could be finished in 28 h. Also, the method were used to detect *B. pseudomallei* in soil samples collected from Guangxi region of China where is the endemic of melioidosis.

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MATERIALS AND METHODS

Bacterial strains

B. pseudomallei CMCC 53001 was provided by National Center for Medical Culture Collection, China (CMCC) *B. pseudomallei* CVCC 67904 and *Burkholderia mallei* CVCC 326 were provided by China Veterinary Culture Collection Center (CVCC). *Burkholderia thailandensis* ATCC 700388 was obtained from the American Type Culture Collection (ATCC). Other bacteria used in this study, including *Proteus spp, Acinetobacter spp, Salmonella typhimurium, Salmonella enterica serovar typhi* CMCC50041*, Bacillus anthracis, Candida albicans, Enterococcus spp, Streptococcus viridans, Streptococcus non-hemolyticus, Staphyloccocus aureus Rosenbach* ATCC25923*, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella enteritidis, Shigella dysenteriae* CMCC51252*, Streptococcus pneumoniae, Neisseria gonorrhoeae, Helicobacter Pylori, Vibrio harveyi, Pseudomonas cepacia* and *Pseudomonas aeruginosa* were isolated from clinical specimens from patients admitted to the first affiliated hospital of the Medical College of Xi'an Jiaotong University. *Escherichia coli* ATCC35150*, Bacillus sphaericus* ATCC11778*, Bacillus thuringiensis* and 13 isolated *B. pseudomallei* from soil samples were laboratory stored. Bacterial culture and DNA preparations of all pathogens were undertaken in biosafety level-2 and 3 Laboratory according to biosafety guidelines.

Cloning and expression of flagellin gene

Chromosomal DNA of *B. pseudomallei* (CMCC 53001) was extracted by a genomic DNA extraction kit (Tiangen, China). Primers 5'-AAAAGAATTCGCGTCGGCGCTGCAACAGGAACTCG-3' and 5'-AAAAAAGCTTTTACATCGCCTGGTACGCGCCCGTCTGC-3' were designed to amplify the truncated flagellin gene by PCR (Chen et al., 2003). The amplified PCR fragment was digested by *Eco*R I and *Hind* III and then introduced into *Eco*R I and *Hind* III-digested plasmid pET 28a (Novagen), a His tag expression vector, resulting in a recombinant plasmid pET-*fli* C. The cloned flagellin gene was then sequenced, and submitted to Genbank (accession number of U73848). Then the recombinant plasmid pET-*fli* C was transformed into *E coli* DE3, and a recombinant *E. coli* strain E- pET-*fli* C was selected and identified. The recombinant flagellin was purified by using a His-Bind Resin chromatography kit following the manufacturer's procedure (Novagen). The protein concentration was determined by a commercial protein assay kit (TIANGEN, China). The purified flagellin was identified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (10% polyacyl-amide gel) and confirmed by western blot analysis (with the rabbit polyclonal antibody of *B. pseudomallei* cells, made in our lab).

Preparation of polyclonal antibodies and Mabs

Two New Zealand white rabbits were immunized by *B. pseudomallei* suspension (10^9 cfu/ml) prepared in Ringer's solution containing 0.3% formaldehyde at doses of 100 mg per rabbit in combination with Freund's adjuvant. Antiserum was collected two weeks after the second boost injection. IgG was purified from the antiserum and labeled with horseradish peroxidase (HRP) for direct ELISA assay.

Ten micrograms of purified flagellin antigen was homogenized in complete Freund's adjuvant and then injected intraperitoneally into BALB/c mice. Two weeks after immunization, the spleens of the immunized mice were collected and the spleen cells were immunized *in vitro* with 1 µg/ml of flagellin antigen. After two days, the cells were fused with a mycloma cell line (P3x63 Ag8.653) according to the procedure as previously described (Pongsunk et al., 1996). Hybrids were screened for antibody production by indirect ELISA. The partially purified MAbs were prepared from the culture supernatants by precipitation with ammonium sulfate and kept in small aliquots at room temperature and -20°C, respectively. Those Mabs were used as capture antibodies in the ELISA assay. Also，Mabs were labeled with horseradish peroxidase (HRP) as detection antibodies for direct ELISA assay.

Double antibodies sandwich enzyme linked immunosorbent assay (DAS-ELISA) method

DAS-ELISA was performed as described previously (van Zijderveld et al., 1992). Briefly, microtiter plates were coated with Mabs as capture antibody. Flagella of *B. pseudomallei* or soil samples were added and washing, HRP-labeled Mabs were added in 200 times dilution. After incubation for 1 h at 37°C and washing, peroxidase substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) was added for 10 min and stopped by the addition of 2 M H_2SO_4 per well. The plates were read at 450 nm with an ELISA reader (Bio-Tek). The optimum dilution of HRP-labeled IgG and Mabs were determined by a titration assay.

Sensitivity and specificity of sandwich enzyme linked immunosorbent assay (ELISA)

To detemine the limit of detection (LOD) of the method, 100 µl of *B. pseudomallei* in a concentration that varied from 10 to 100,000,000 CFU/ml were tested by the DAS-ELISA method. To detect the specificity and sensitivity of the method, all bacterial strains kept in our lab were cultured in LB broth 37°C 24 h. 100 µl of each was used for the test.

Detection of *B. pseudomallei* in soil samples collected in endemic area

Totally 154 soil samples were collected from 77 sampling sites mainly in the rice field in winter in December 2007 in Guangxi province where is the endemic of melioidosis in China. Approximately 10 g of soil sample was obtained at a depth of 30 to 60 cm from the bottom of each hole and placed into a sterile plastic bag, and each site was sampled from two separate holes at the same time. All the samples had been identified by the classical culture method in 2008 (Guard et al., 2009). All the soil samples were kept in 4°C.

One gram of the soil sample was added to 9 ml of LB broth plus colistin and gentamicin (100 µg/ml each), and the mixtures were incubated at 37°C for 24 h. 100 µl of cultural broth was detected by the sandwich ELISA.

RESULTS

Cloning and expression of flagellin gene

The amplified truncated flagellin fragment was sequenced, and the result was submitted to NCBI for alignment against nucleotide databases (Accession Number: U73848). The truncated flagellin was expressed in *E. coli* BL21 (DE3) after induction of IPTG, and purified by using a His-Bind Resin chromatography kit (Novagen). SDS-PAGE showed that purified flagellin revealed single protein band with an

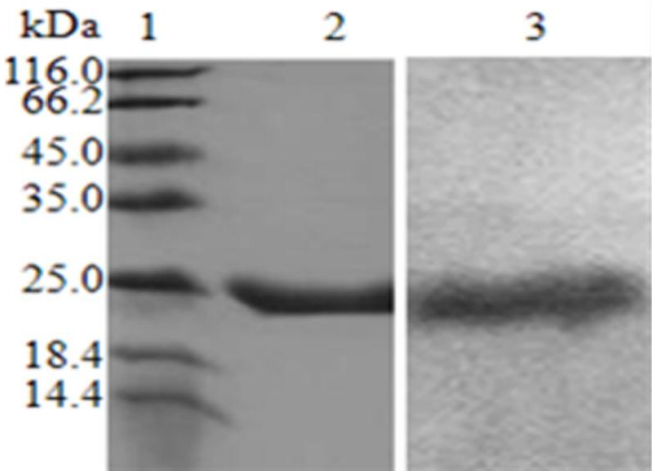

Figure 1. SDS-PAGE and western blotting of the recombinant flagellin. Lane 1: low molecular weight markers; Lane 2: purified recombinant flagellin; Lane 3: western blotting of the flagellin.

estimated molecular weight 22 kD (Figure 1). Western blotting showed that purified flagellin was reactive with the antiserum to *B. pseudomallei* (Figure 1).

Characterization of Mabs and polyclonal antibody

Ten hybridomas, secreting antibody reactive with flagellin antigen of *B. pseudomallei*, were obtained. The titers of Mabs were detected with an indirect ELISA which coated with the whole cell bacterial antigen 5 ug per well. The titer of the Mab 3 and Mab 5 reached to 1:400,000 to the whole cell bacterial antigen. Mab 3 was used as capture antibody in the DAS-ELESA assay to detect *B. pseudomallei* in soil sample (Table 1). Mab 5 was marked with HRP as detection antibody in the ELISA assay.

The specificity and sensitivity of the Double antibodies sandwich enzyme linked immunosorbent assay (DAS-ELISA) assay

DAS-ELISA method was used to detect different bacterial strains kept in our lab. The result indicated that all positive samples gave OD_{450} values above 3.000, while negative samples below 0.500. The cutoff value was calculated to be 0.500, about 2 times of the OD_{450} value of *Pseudomonas cepacia*, which had a week reaction with the Mab (Table 2).

The results show that the 15 isolates of *B. pseudomallei* were positive. However, *B. mallei*, which has almost the same genotype with *B. pseudomallei* and is difficult to distinguish by ELISA, did not react with the Mabs in the ELISA assay; also, the method could differ *B. thailandensis* from *B. pseudomallei* (Table 2). Cultured *B. pseudomallei* broths were used to estimate the limit of detection (LOD) of the ELSIA. The LOD of the ELISA assay was 1.0×10^5 CFU/ml (Figure 2).

Detection of *B. pseudomallei* in soil samples in endemic area

The occurrences of *B. pseudomallei* in 154 soil samples were analyzed by sandwich ELISA and the gold culture method. Of all 13 positive samples isolated from different place in endemic area, 13 were detected positive by the DAS-ELISA method (Table 3). The results of ELISA and culture method were greatly consistent. The results show that the sensitivity and specificity of the ELISA method is 100% (13/13).

DISCUSSION

The flagellin gene of *B. pseudomallei* is specific, therefore, it were used to identify *B. pseudomallei* by PCR (Sonthayanon et al., 2002; Tomaso et al., 2005). But *B. mallei* also has the flagellin gene, which is the same as *B. pseudomallei*. It is difficult to distinguish *B. pseudomallei* from *B. mallei* by PCR methods based on flagellin gene. So the antibodies of *B. pseudomallei* to flagellin could identify *B. pseudomallei* from *B. mallei*. The gene of FliC of *B. pseudomallei* has 84% similarity with *B. thailandensis,* therefore, flagellin could be used to differ *B. thailandensis* from *B. pseudomallei*, Flagellin elicit early specific antibodies against *B. pseudomallei* infection in mice. It is an ideal antigen for detecting melioidosis. Flagellin were used as a molecular probe for diagnosis of melioidosis, and achieved 93.8% sensitivity and 96.3% specificity (Chen et al., 2003). Here, we used the truncated flagellin of *B. pseudomallei* as an antigen to immune BALB/c mice, and got high specificity monoclonal antibodies and developed the DAS-ELISA assay to identify *B. pseudomallei.*

The sensitivity of the ELISA assay for *B. pseudomallei* culture broth was 1.0×10^5 CFU/ml (Figure 2). However, in studies conducted in Laos and Thailand, environmental *B. pseudomallei* load was assessed by using quantitative culture of soil samples. A concentration range of 10 to 1,200 CFU/g soil with a geometric mean of 39 CFU/g soil was found for the laotian samples and a median of 10 and 230 CFU/g soil for central and North-east Thailand, respectively (Kaestli et al., 2007). Also in a China latest study, the concentration of *B. pseudomallei* in Guangxi province ranges from 20 to 1000 CFU/g soil sample (Guard et al., 2009). Therefore, the DAS-ELISA could not be used to directly detect *B. pseudomallei* in soil samples.

In order to detect *B. pseudomallei* in soil samples and avoid false negative detection, the selective culture broth of *B. pseudomallei* plus colistin and gentamicin were used to propagate the *B. pseudomallei* colony, which has

Table 1. Antibody titers to the whole cell bacterial antigen.

Antibody	1	2	3	4	5	6	7	8
	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600	1:51,200	1:102,400	1:204,800
MAb 3	1.014	0.926	0.910	0.852	0.544	0.519	0.364	0.233
MAb 5	1.212	1.012	0.906	0.805	0655	0.547	0.348	0.332

Table 2. Reactivity of the DAS-ELISA with different bacterial strains.

Genera	Species	No. tested	No. positive	ELISA (OD_{450})
Bulkholderia	*B. pseudomallei*	15	15	3.225 ± 0.100
	B. thailandensis	1	1	0.043± 0.210
	B. mallei	1	0	0.034 ± 0.006
Bacillus	*B. anthracis*	2	0	0.014 ± 0.004
	B. thuringiensis	3	0	0.014 ± 0.006
	B. sphaericus	1	0	0.013 ± 0.003
Candida	*C. albicans*	1	0	0.023 ± 0.009
Enterococcus	*E. faecalis*	1	0	0.023 ± 0.010
Escherchia	*E. coli*	2	0	0.013 ± 0.008
Helicobacter	*H. pylori*	1	0	0.012 ± 0.005
Neisseria	*N. gonorrhoeae*	1	0	0.025 ± 0.008
Proteus	*P. mirabilis*	1	0	0.023 ± 0.006
Pseudomonas	*P. aeruginosa*	1	0	0.043 ± 0.007
	P. cepacia	2	0	0.225 ± 0.008
Shigella	*S. dysenteriae*	1	0	0.028± 0.005
Salmonella	*S. enterica*	1	0	0.029 ± 0.005
	S. enteritidis	1	0	0.027 ± 0.006
	S. typhimurium	1	0	0.028 ± 0.004
Staphylococcus	*S. aureus*	1	0	0.045 ± 0.004
	S. aureus Rosenbach	1	0	0.026 ± 0.005
	S. epidermidis	1	0	0.032 ± 0.008
Streptococcus	*S. pneumoniae*	1	0	0.023 ± 0.007
	S. viridans	1	0	0.034 ± 0.004
	S.non-hemolytic	2	0	0.026 ± 0.004
Vibrio	V.harveyi	1	0	0.036 ± 0.006
Negative control		4	0	0.010 ± 0.002

Average OD_{450} for three repetitions and the arithmetic mean ± SD is given.

a good inhibition reaction to most of other bacteria. However *B. pseudomallei* colonies could survive and propagate in that broth (Walsh and Wuthiekanun, 1996). The ELISA was used to detect 154 soil samples collected from Guangxi section where there is the endemic of melioidosis in China. 13 soil samples were detected positive. It was the same as the culture method.

Compared with culture, which is the gold standard for *B. pseudomallei* detection in soil, a week in detection is needed (Novak et al., 2006); the sandwich ELISA assay method needs only 28 h in detection test. There were many methods for detection the blood culture fluid from

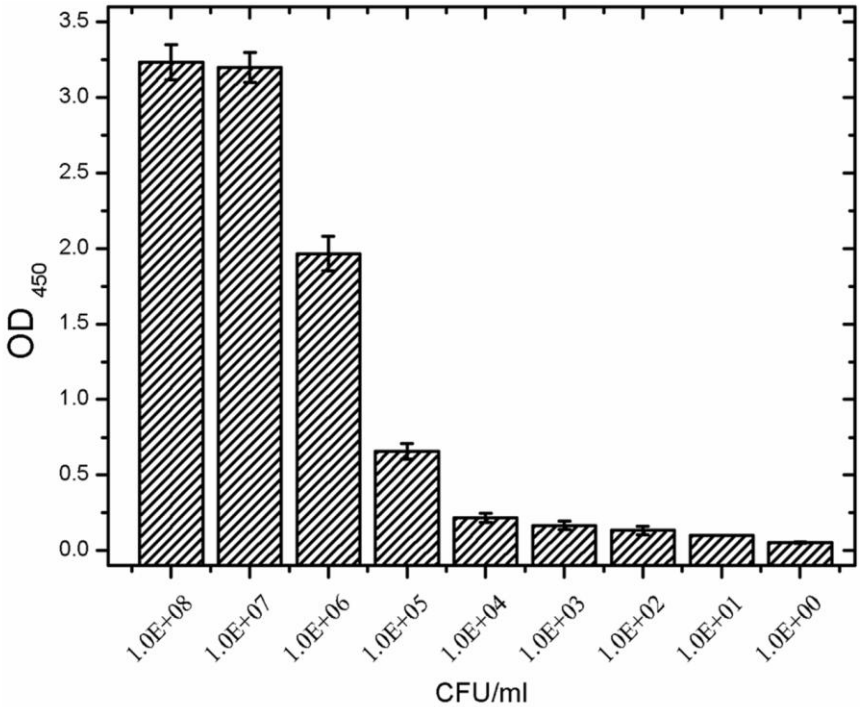

Figure 2. The LOD for the detection of *B. pseudomallei.* X axis was the concentration of the bacteria (CFU/ml), Y axis was the OD_{450} values. The LOD was determined to be 1.0×10^5 CFU/ml in accordance with the cutoff value of 0.400.

Table 3. Detection comparison of *B. pseudomallei* in soil by the DAS-ELISA and culture method.

Soil sample	DAS-ELISA		Culture	Bacterial load (CFU/g soil)
	OD_{450}	Result		
Zhongdong1	1.234± 0.011	+	+	337
Zhongdong2	1.342 ± 0.012	+	+	402
Nanning1	1.432 ± 0.009	+	+	103
Nanning2	0.987 ± 0.006	+	+	73
Nanning3	1.143 ± 0.007	+	+	27
Nanning4	1.566 ±0.006	+	+	203
Qinzhou1	1.456 ±0.012	+	+	189
Qinzhou2	1.432 ±0.013	+	+	521
Qinzhou3	1.456 ±0.004	+	+	23
Fangcheng1	1.578 ±0.008	+	+	354
Fangcheng2	1.723 ±0.008	+	+	267
Beihai1	1.678 ±0.006	+	+	431
Beihai2	1.032 ±0.010	+	+	206
Negative control	0.015 ± 0.002	-	-	0

+ positive, - negative; Average bacterial load of three repetitions; Average OD_{450} for three repetitions and the arithmetic mean ± SD is given.

patients by ELISA (El-Masry et al., 2008; Escobar-Gutierrez et al., 1996; Sermswan et al., 2000). Few of ELISA was used to detect the soil cultures. However the sandwich ELISA is available for detection of *B. pseudomallei* directly in soil culture. It is applicable for large screening studies to assess the prevalence of *B. pseudomallei* in soil. It also can be used to detect the potential source of contamination in outbreak situations of melioidosis.

ACKNOWLEDGEMENTS

We are grateful to Yan bing for helpful comment and suggestions in the ELISA test. This project was partially supported by a grant (24GFCX-YJ-2306) from Chinese Academy of Sciences and National S&T Major Project (2008ZX10004-010, 2009ZX09301-14).

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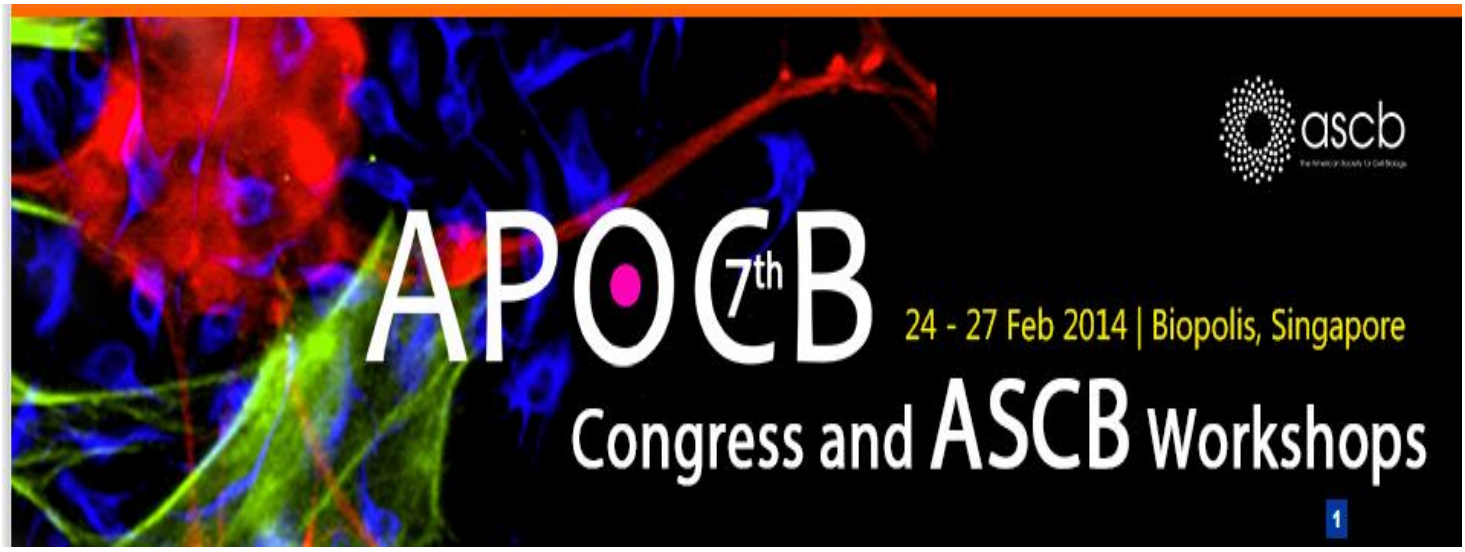

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