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ORIGINAL ARTICLE

ADOLESCENT'S ATTITUDES TOWARDS HEALTH WARNING MESSAGE ON CIGARETTE PACKS

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A total of 190 secondary four male school students from three schools in Kota Bharu were surveyed on their smoking habits and their attitudes towards the health warning messages on cigarette packs. There were 57 (30.0%) students who were current smokers, 45 (23.7%) students who were ex-smokers and 88 (46.3%) students who have never smoked cigarettes. Nearly all current and ex-smokers (95.1%) as well as non-smokers (94.3%) knew the wording of the health warning message currently displayed on cigarette packs. Almost all the students (95.3%) also knew where the warning message was placed. There were more ex-smokers and non-smokers (70.5%) compared to current smokers (50.0%) who felt that there should be different health warning messages and each should be displayed concurrently on different cigarette packs. The students felt that the current health message was not effective to motivate smokers to quit (score=2.25). Alternative messages which the students felt may be more effective were 'Smoking is dangerous for pregnancy' (score = 3.3), 'Cigarette smoke is dangerous for your child' (score=3.11) and 'Smoking can kill you' (score=3.08). The current health message " Smoking is dangerous for your health' is eighth with a score of only 2.64. The students felt that the least effective message was 'Cigarettes are drugs' (score=2.22). Most of the students (80.0%) felt that the health warning message should be placed at the front instead of on the side of the cigarette pack to be more effective.

Key words : *smoking, health messages, adolescents, Kota Bharu*

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Introduction

Smoking among adolescents has been found to be a growing problem in many developing countries (1). Traditionally, smoking control has focused on educating smokers or would be smokers on the dangers of smoking. Health warnings on cigarette packs are mainly utilised to educate smokers and is a useful and inexpensive way to reach smokers. To be effective, health warnings should be able to motivate smokers to quit. It is usually assumed that, because health warnings are so important, smokers will find this information so interesting and convincing, that, after reading it, they will quit smoking. This does not happen generally and their efficacy is limited (2). There is a need to actively market these messages on cigarette packs to help diminish the impact of cigarette advertising.

In Malaysia, the Control of Tobacco Product Regulations 1993 has been enacted to reduce tobacco consumption in the country, especially among youth. The regulations requires the tobacco companies to have a health warning message 'Smoking is dangerous to your health' in the local language on cigarette packs. However, it is generally felt that the message and the way it is displayed, is not very effective to motivate smokers to quit, especially adolescents. The problem of 'wearout' by using the same health warning message is a well known problem (3). Secondly, for the youth, who are at their prime of health, the perception of being 'dangerous to health' has only a very limited impact (4,5). To

prevent 'wearout' and to target the message to various target groups, different messages rotated between cigarette packs is recommended (6). The health warning message is presently placed on the side of the cigarette pack in relatively small print. The background colour is the same as the colour of the cigarette pack, making the message almost invisible. Ideally, the health warning message should be the first thing smokers see before buying the cigarettes and the last thing they see before lighting up the cigarettes (7).

This study is an attempt to assess the attitude of adolescent Malaysian school students on the effectiveness of the current mandatory health warning message on cigarette packs. The perception of these adolescents to the effectiveness of alternative health warning messages and the most effective place for the message on the cigarette pack was also done.

Methodology

Three secondary schools were randomly sampled from the Kota Bharu district. All male secondary four students in the selected schools were included in the study. However, after discussions with the teachers, it was felt that only students from certain classes were able to read and understand the questionnaire, thus providing a more reliable response. All male students from these classes who were present on the day of the study were selected and participated in this study. A total of 190 secondary four male school students were studied.

Each student was given a questionnaire which were collected at the end of the session. The questionnaire was in Malay to improve the student's understanding and response. Twelve alternative health warning messages in Malay were given to the students. Ten of these alternative health warning messages were health warning messages from Canada while one was the message found in Malaysia. The last alternative given was based on the religious belief that smoking is forbidden. The students were asked to give a score on their perceived effectiveness of these messages to motivate a smoker to quit. The current health warning message 'Smoking is dangerous to your health' was also included to compare the score with that of the other messages. Another alternative health warning message 'Smoking is forbidden (haram)' was also included to assess the potential effectiveness of using religious warnings. The students were asked to score the potential effectiveness of these health warning messages on a 5 point Likert scale: 1=not effective, 2= mildly effective, 3=moderately effective, 4=quite effective 5=very effective. To improve the reliability of the result, the questionnaire was anonymous. The 'bogus pipeline technique', using sampling of each student's saliva, was used as this method is known to improve the accuracy of self reported current smoking habits (8).

The data was analysed by t-tests and analysis of variance using Epi Info Version 6, a word processing, database and statistical software for public health, (9).

Results

There were 57 students who were current smokers, giving a smoking prevalence of 30.0%. There were 45 students (23.7%) who are ex-smokers and 88 students (46.3%) who have never smoke cigarettes. Therefore, a total of 102 students (53.7%) have been exposed to smoking cigarettes, either currently or previously.

Nearly all the students, including both current and ex-smokers (95.1%) and non-smokers (94.3%) were able to write down the current health warning message found on the cigarette packs. Again, nearly all of them (95.3%) knew the site of the health warning message on the cigarette pack. More non-smokers and ex-smokers (70.5%) compared to smokers (50.0%) felt that there should be alternative health warning messages used on cigarette packs. One hundred and fifty-two students (80.0%) felt that the health warning message should be in front of the cigarette pack for it to be more effective. Only 5 students (2.6%) chose the current site, which is at the side of the cigarette pack, as the best site for the health warning message.

Almost all (84.2%) of the current smokers regular read the health warning message found on cigarette packs. There was no significant difference in the score of the effectiveness of the current health

Table 1. Effectiveness of current health warning message by Form 4 male students

Smoking Status	Score of effectiveness					
	1	2	3	4	5	Average
Current smoker	11	14	12	8	12	2.93
Ex-smoker	12	13	13	3	4	2.42
Non smoker	24	24	20	7	13	2.56
Total	47	51	45	18	29	2.64
F statistic = 2.068, p > 0.05						

warning message ' Smoking is dangerous to your health' among the three categories of students according to their smoking status. (Table 1)

The mean score on the potential effectiveness of these eleven alternative health warning messages are shown in Table 2. The message 'Smoking during pregnancy can harm your baby' and 'Tobacco smoke can harm your children' obtained the highest scores, with mean scores of 3.26 and 3.11 respectively. The messages 'Cigarettes are addictive' and 'Smoking is forbidden' were scored the least effective with mean scores of only 2.33 and 2.22 respectively.

A comparison on the mean score between current smokers with non-smokers and ex-smokers indicate that generally, the smoker's score was higher compared to the non-smoker and ex-smoker's score. However, there were only five health warning messages in which the score was significantly higher for the current smokers. The score for the religious based health warning message 'Smoking is haram' is higher for non and ex-smokers compared to smokers although the difference was not significant.

Table 2. Score of effectiveness of alternative health messages by category of smoker

Health warning message	Mean Score			p value
	All	Smokers	Non / Ex	
1. Smoking during pregnancy can harm your baby	3.26	3.35	3.22	n.s
2. Tobacco smoke can harm your children	3.11	3.39	2.99	< 0.05
3. Smoking can kill you	3.08	3.02	3.11	n.s
4. Cigarettes cause fatal lung disease	3.06	3.30	2.88	< 0.05
5. Cigarettes causes cancer	2.98	2.88	3.02	n.s
6. Cigarettes causes stroke and heart disease	2.68	2.72	2.68	n.s
7. Smoking causes bad breath and yellow teeth	2.64	3.07	2.46	< 0.01
8. Smoking is dangerous to your health	2.64	2.93	2.51	n.s
9. Smoking is a waste of money	2.58	3.19	2.32	< 0.01
10. Tobacco smoke causes fatal lung disease in non-smokers	2.52	2.81	2.39	< 0.05
11. Smoking is haram	2.33	2.15	2.55	n.s
12. Cigarettes are addictive	2.22	2.21	2.22	n.s

Discussion

The current requirements for the tobacco companies to place warning signs on cigarette packs in Malaysia should be changed to increase it's effectiveness to encourage smokers to quit smoking. The size, location, colour and content of these health warning messages should be considered. This is based upon research findings, which indicates that changing any one of these parameters will help bring the messages out of the noise (10,11). Bhalla and Lastovicka have also concluded that the more severe the departure of the format from the existing format and the less textual the advertising context, the greater the potential effect (12).

In this study, most of the students were able to correctly write down the current health warning message, and the site where it is situated. However, nearly all of them felt that the message is not effective to motivate smokers to quit. Most of the students agree that there should be different messages and should be placed in front of the cigarette pack. Studies have found that adolescent smokers are more attentive than adults, especially to rotating meaningful messages (13,14). The messages can also be used to target different groups of smoker. Messages about smoking affecting the looks of the smoker may be more meaningful to the teenage smoker, which was found to be significantly more in smokers. The message on smoking harming children was also significantly more in smokers. However, this message may be more meaningful to adult smokers who have children. The religious

health warning message given in this study was less meaningful to these adolescents. Religion may not be a major concern at this age but such religion based messages may have a better impact among the older smokers.

There are a number of countries like Australia, Canada, Norway, Singapore and Thailand, who are leaders in the area of health warning messages. During the mid-1990s, these countries had introduced health warning messages with much greater impact, including direct statements of health hazards and multiple messages, that were larger and more prominently displayed (3). This will provide a more effective and inexpensive way, from the Government's point of view, of reaching and educating smokers. In Canada, the colour of the warning signs is displayed by bold black letters on a white background, which stands out against the background colour of the cigarette pack. The message is placed prominently at the front of the pack. The front of the cigarette pack is the largest and visible display surface and the message should occupy the top 25% of the surface. Cigarette manufacturers in Canada are required to ensure that all the messages appear simultaneously, with half of their packaging containing a warning in black text on a white background and the other half in white text on a black background (7).

Mandatory health messages on cigarette packages is a useful, effective and inexpensive way of reaching the smoker. However, the purchasing habits of the child and adolescent smoker is different from the adult smoker. They are known to usually purchase individual cigarettes instead of buying a whole pack (15). This habit will render the health warning messages on the cigarette packs ineffective. Experience in other countries has shown that banning the sale of individual cigarettes may reduce the problem (16). Selling cigarettes only in packs will limit the accessibility of children and adolescents to cigarettes as they are more sensitive to the price of a pack of cigarettes (17,18). In Malaysia, this additional requirement is essential to boost the effectiveness of the mandatory health warning message found on cigarette packs.

Mandatory health warning message on cigarette packs is only one component of a comprehensive tobacco control strategy. Other components include a total ban of cigarette advertising, effective protection from involuntary exposure to tobacco smoke, high tobacco taxes, a ban of incoming duty free sales of cigarettes, a prohibition of sales to minors and vigorous health promotion activities. All these together have contributed to the spectacular decline in cigarette sales and a radical change in the marketing of tobacco products in those countries which are successful in controlling tobacco consumption (19).

Conclusion

Our study confirms the need to review and enhance the existing mandatory health warning messages on cigarette packs in Malaysia. The size, location, colour and content should be changed to enhanced it's effectiveness. Hopefully, combined with other control measures, Malaysia could very soon enjoy the same success in curbing tobacco consumption that other countries has through their comprehensive tobacco control program.

Acknowledgements

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ORIGINAL ARTICLE

OBSERVATIONAL STUDY ON CANNULATION RATE DURING ERCP AT HOSPITAL ALOR SETAR

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ERCP (Endoscopic Retrograde Cholangiopancreaticography) was introduced in this hospital in late 1995.Since then, a lot of improvement have been achieved in the management of biliary tract diseases. Various general surgeons posted to this hospital have been trained in this procedure. A study was done to include all patients admitted for ERCP from August 1998 to July 1999. A total of 322 new patients with a mean of 26.9 cases a month had underwent this procedure. The duration of cannulation varied from 2 minutes to 45 minutes with a mean of 12 minutes. Cannulation rate by various surgeons differed. Overall success rate was 80%. Mortality was 0.6 % and morbidity was 0.9%. ERCP is safe and it takes at least 6 months of regular duodenoscopy before one can master the technique. Achieving 80% cannulation rate, has definitely reduced unnecessary common bile duct (CBD) explorations. During this study we have trained various surgeons in this procedure and at least 2 surgeons could be credentialled according to the guidelines provided by the Malaysian Society of Gastroenterology and Hepatology. During this study we have identified various reasons for the failure of cannulation which are useful for future training of endoscopists.

Key words : *Cannulation rate, ERCP*

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Introduction

Endoscopic retrograde cholangiopancreaticography (ERSP) was first described by Mc Cune et al in 1968 but was later popularized by Oi et al in Japan, as well as Demlin and Classen in Europe in 1970's as a diagnostic tool (1,2). In 1974, Classen and Demlin performed the first endoscopic sphinterotomy and since then this procedure has been used for stenting, stone crushing and nasobiliary drainage.

In Malaysia, facilities to perform this procedure was only available 10 years ago. Hepatology and hepatobiliary surgery more specialist are being trained in this field.

In Hospital Alor Setar, none of the surgeons except one ,has formally been trained in this. The success of ERCP depends on how well one cannulates the major duodenal papilla. Further therapeutic work only follows after this step. This study was designed to assess the successfullness of ERCP and the it's shorfalls in a non subspecialized hospital like Hospital Alor Setar.

Materials and Methods

322 new patients come for ERCP procedure for various indications from August 1998 to July 1999 were included in this study. After an overnight fasting , they were given i.v cefuroxime 1.5 gm stat in the ward prior to the procedure. Protrombin and activated partial tromboplastin time were corrected to near normal levels in all patients.

The procedure was performed with the patient

in the prone position. All patients were monitored with a pulse oxymeter. Initial sedation was with 3 mg midazolam and 30 mg pethidine.I.V hyoscine was only given if the initial pulse was less than 120/ min. Further additional sedation was given if necessary.

The time of cannulation was taken from the time of introduction of the duodenoscope and the time of successful CBD cannulation detected on fluoroscopy. After completion of this procedure, whether for diagnostic or therapeutic purposes, findings were documented into a special performa form.

All surgeons who did this procedure had performed at least 100 upper gastrointestinal scopes (OGDS). Every surgeon was given between 10 to 20 minutes , failing which a rescue surgeon took over.In this hospital, time allocated for ERCP was 5 hrs 30 min per week.

Results

Demographic data shows a male to female ratio of almost 1:1.Malays being the main racial group in this part of the country constituted 80% of the cases.

Figure 2 showed that, the majority of the patients were in the age group of 50 to 64 years .The youngest was 13 years who came for CBD injury after lapascopic cholecystectomy . He had a hemolytic disorder presenting with gallstones. The oldest was 90 years presenting with large CBD stones.

Indications for ERCP during the study are shown in Table 1. The biodata of the surgeons are briefly indicated in Table 2.

Figure 3 to 7 indicates the performance by various surgeons who have been handling this procedure in order to gain experience. Figure 3 showed that the trainer had a good success rate although since October 1998, he did less procedure in anticipation of his transfer.

Figure 4 showed the results of a regular performer of this procedure .It took a minimum of 75 cases to reach competence level which was above 80%.

Unfortunately most of the other doctors did not perform the procedure frequently as in figure 5, but there was a sign that these doctors were showing some improvement from March 1999. The overall cannulation rate was reduced below 90% due to poor

Figure 1. Racial distribution

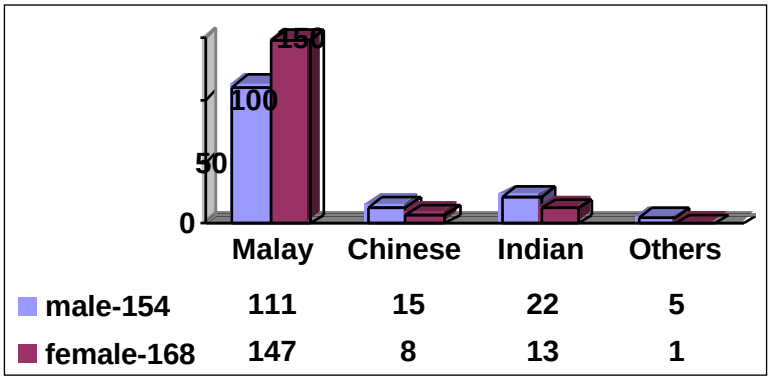
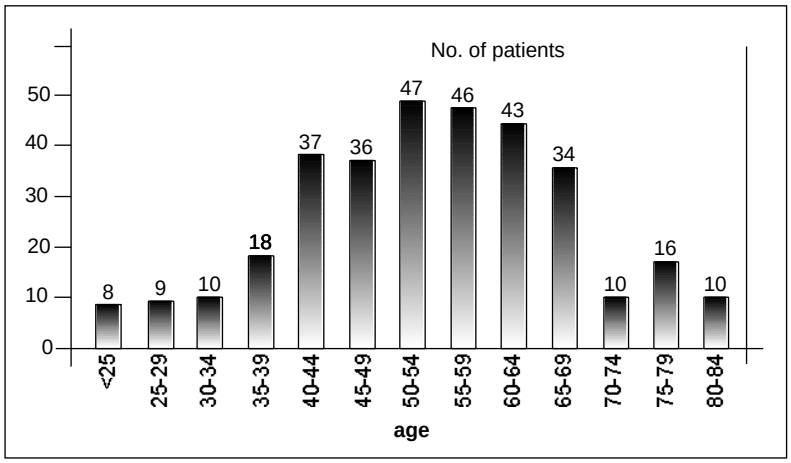


Figure 2. Age Distribution of the patients



cannulation.

The overall success rate for this procedure was good (> 80%) except in Oct 98,Nov 98,Apr 99 and Jun 99 where the rescue doctors were on frequent leave. The total overall cannulation rate was 79.9 % during this study. The value would have been higher if a few cases where the ampulla was not seen or even destroyed by periampullary carcinoma had been excluded.

We also looked into the reasons why the cannulation was not successful. The various reasons ranged from cannulating from far, inability to centralize the ampulla, inability to coordinate the small and large wheels of the duodenoscope, insufflation of too much air into the stomach leading to the inability to enter the pylorus, ampulla in a duodenal diverticulum, flat ampulla, ampulla hiding in the hood of duodenal folds, not well versed with the precut technique, as well as tumour and stone blocking the lower CBD.

There were two morbidities, one developed acute pancreatitis and the other cholangitis and two morctulities, one died due to acute pancreatitis and the other due to cholangitis.

269 patients (83%) had endoscopic sphincterotomy and therapeutic intervention at the same time while the rest 17% had diagnostic procedure. The above figures give us an overall mortalities of 0.9% and mortality of 0.6% . A part from the oesophageal perforation , our complications were due to sphincterotomy. This figure might be biased because we did not routinely do serum amylase level after ERCP. It was found that up to 75% of the cases could be associated with painless hyperamylasemia which did not need any treatment. (3) Acute pancreatitis is a known dreaded complication and the incidence reported varies between 0.7% to 7.4%.In our study it was 0.6% and one patient died of severe haemorrhagic pancreatitis. Our oesophageal perforation occurred due to inability of a new surgeon to manipulate the scope through the cricopharyngeus in a frail old lady who subsequently died of mediatinitis. Although oesophageal perforation is a known complication of endoscopy especially with the side viewing duodenoscope, there is no data on the incidence rate. Such incidence have been reported only once (5).

Conclusion

The above study has clearly showed that ERCP is not a procedure, which can be performed easily even by surgeons with a wide experience in endoscopy. Surgeons who are beginning to perform ERCP need a lot of patience and more importantly one should continuously practice doing this procedure with guidance .It took doctor B , six solid months of training before he could reach 90% success in cannulating the major duodenal papilla.Although this is a good guide but this is only a single surgeon's figure .Since we have identified our basic shortfalls , we hope that better training can be provided for new surgeons.

Referring to the recommendations of the Malaysian Society of Gastroenterology and Hepatology (4) , a unit accredited for training in ERCP should perform a minimum of 150 ERCP's a year.

Table 1 :- Indications for ERCP

Biliary System	
Choledocholithiasis	172
Obstructive jaundice	34
Acute cholangitis	16
Cholelithiasis with of jaundice	16
Intrahepatic stone	6
Cholangiocarcinoma	6
Raised alkaline phosphatase	3
Acute cholecystitis	3
CBD injury	3
Jaundice for investigation	2
Choledochal cyst	1

Pancreas	
Acute Pancreatitis	30
Chronic Pancreatitis	9
Carcinoma Head of Pancreas	11
Carcinoma Ampulla of Vater	6
Carcinoma Body of Pancreas	1
Pancreatic Pseudocyst	2
Polyps on Ampulla of Vater	1

Table 2. Biodata of Surgeons

a) Surgeon A-		Consultant. Trained in ERCP. Started this service. Frequent participation. Transferred out in Jan.1999
b) Surgeon B-		Clinical specialist.On the job training. Frequent participation
c) Others	-	Other consultants, Clinical specialist, visiting specialist. Infrequent participation
d) Rescue	-	Surgeon A +/- Surgeon C

Figure 3. Surgeon A

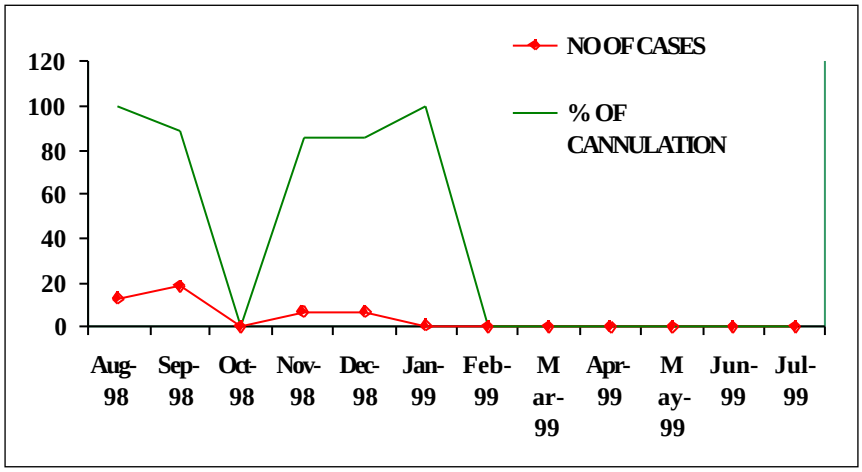


Figure 4. Surgeon B

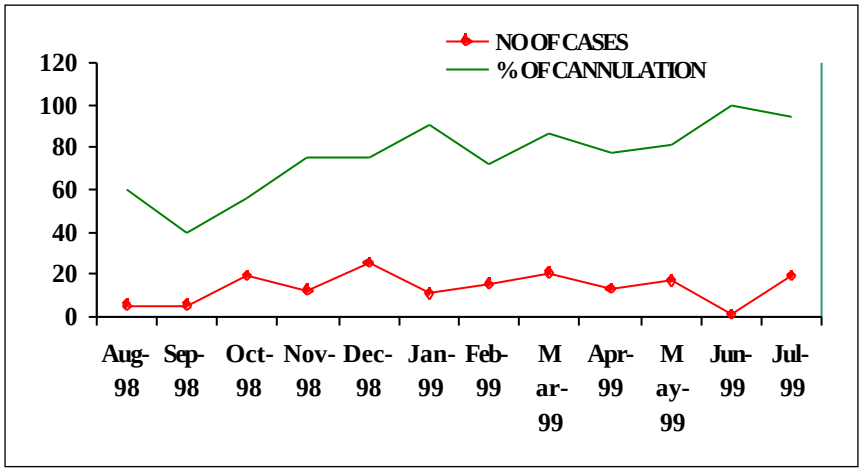


Figure 5. Others

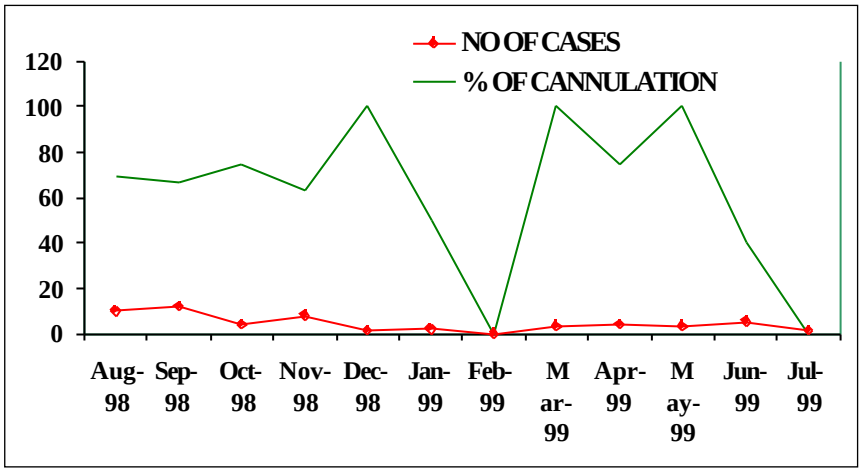


Figure 6. Rescue

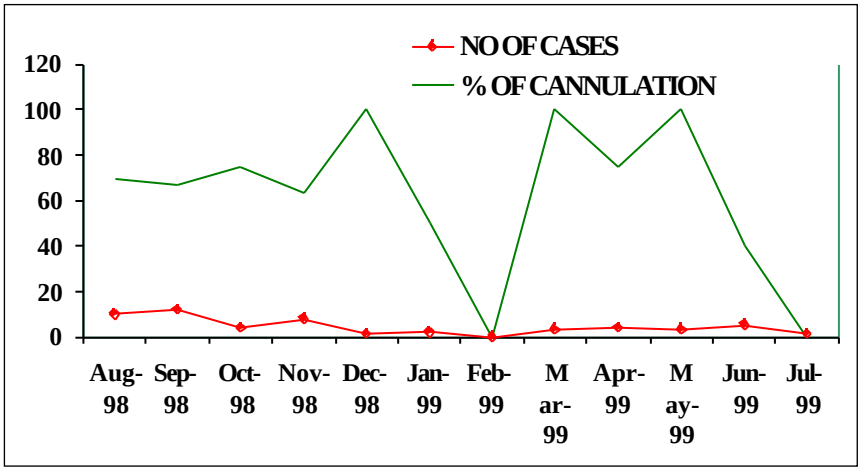
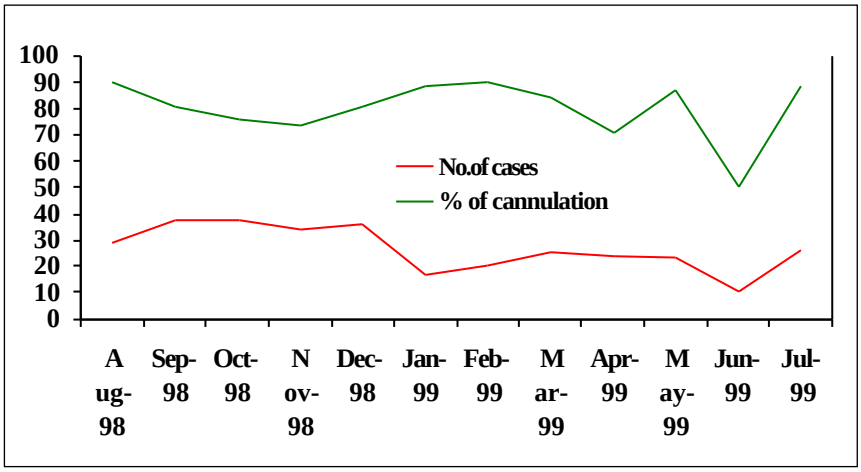


Figure 7. Overall Success



Looking into the credentialing requirements by this society , we have at least 2 surgeons who during this period of the study have been successful in reaching this figure.

Finally this study has shown that, ERCP is a relatively a safe procedure if it can be done with proper supervision. The success in ERCP services have definitely reduced the number of open common bile duct surgery and with a proportional increase in laparoscopic cholecystectomy procedures being done.

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ORIGINAL ARTICLE

PRELIMINARY STUDY SUGGESTS LOW INCIDENCE OF GASTRIC CARCINOMA IN KELANTAN RELATES TO LOW RATE OF *HELICOBACTER PYLORI* INFECTION

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***Helicobacter pylori*-associated gastric carcinoma is generally more common in the antrum/body and is of the intestinal type. The aim of this study was to determine the pattern of gastric carcinoma in an area known to have a low prevalence of *H. pylori*. Pathology records of gastric carcinoma diagnosed at Hospital University Sains Malaysia between 1995 and 1999 were retrieved and studied. There were a total of 23 cases. The median age was 60 years. Eighteen patients were Malay and 5 were Chinese. The most common location of the tumour was the cardia/gastro-oesophageal junction (61%, 14/23 patients). The majority was of the intestinal type (69.6%, 16/23). The frequency of gastric carcinoma appears to be exceptionally low in the area of study. The Chinese population was over-represented. The higher frequency of tumour in the cardia/gastro-oesophageal junction as compared to the antrum and body is in sharp contrast to most other studies. This reaffirms the notion that *Helicobacter pylori* infection is a causative agent for non-cardia gastric carcinomas.**

Key words : *gastric carcinoma, low H. pylori prevalence*

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Introduction

Cancer of the stomach is the second most common fatal malignancy in the world. Its incidence varies worldwide, being high in Japan and considerably lower in the United States, United Kingdom and Canada. It is ranked fourteenth among all primary cancers in Hospital University Sains Malaysia during the period 1995 to 1999.

There are many aetiological factors associated with gastric carcinoma. The incidence of *Helicobacter pylori* closely parallels that of gastric carcinoma and it is considered to be an essential co-factor in the pathogenesis of the intestinal type of gastric carcinoma (1). *H. pylori*-associated gastric carcinoma is generally more common in the antrum and body as compared to the cardia (2,3).

Kelantan is reported to have one of the lowest prevalence of *H. pylori* infection in the world (4). With this unique epidemiological pattern the main aim of this study was to compare the local pattern of gastric carcinoma to that reported in the literature.

Materials and Methods

The pathology records of gastric carcinoma diagnosed at Hospital USM, Kota Bharu between 1995 and 1999 were retrospectively reviewed. The biographic data of patients with reference to age, sex and racial group were documented. Further information as to the type of specimen received at the laboratory, the site of gastric carcinoma and its histological type according to the modified Lauren classification were determined. Biopsy sections were not examined for *H. pylori.*

Results

A total of 964 gastric biopsies and gastrectomy specimens were received at the laboratory between 1995 and 1999. There were 23 cases of gastric carcinomas (19 males, median age 60 years and range 49 to 86 years) diagnosed based on sixteen gastric biopsies and seven gastrectomies. The ethnic composition consisted of 18 Malays and 5 Chinese. The majority of the tumours were located in the cardia/gastro-oesophageal junction (14/23, 61%) while 5 tumours were in the body and 4 in the antrum. All tumours were adenocarcinomas. The most common histological type of gastric carcinoma according to the modified Lauren classification was the intestinal type (16/23, 69.6%) with 5 cases (21.7%) of diffuse type and 2 cases of mixed type.

Discussion

The incidence of gastric carcinoma appears exceptionally low in our institution and is further substantiated by another recent study conducted in Kelantan where the calculated incidence rates for gastric carcinoma in 1997, 1998 and 1999 were 1.4, 1.2 and 1.2 per 100,000 population respectively (5). *Helicobacter pylori* has been classified by the International Agency for Research on Cancer (IARC) as a group I carcinogen (1). The hypothesis is that *H. pylori* causes chronic atrophic gastritis and intestinal metaplasia which progresses to gastric dysplasia and carcinoma. Therefore it is not surprising that the incidence of gastric carcinoma closely parallels that of *H. pylori* infection rates.

The median age of our gastric carcinoma patients of 60 years is comparable to most other series. Gastric carcinoma is known to be more common in males. According to hospital statistics, Chinese represented less than 9% of the annual number of inpatients and outpatients during the study period. It is obvious that this ethnic group is over represented in this series (22%) although the number of cases is small. The ethnic disproportion was also reflected in the study conducted in Kelantan in which Chinese formed 19.3% of patients with gastric carcinoma (5).

In most series where the *H. pylori* infection rates are high, gastric carcinoma is most often located in the non-cardia location (2,6). However in our study 61% (14/23) of cases had tumour in the cardia/gastro-oesophageal region. This finding was reaffirmed in the Kelantan study in which 71% of tumours were located in the proximal part of the stomach (5). In another study that looked at *Helicobacter pylori*-seronegative gastric carcinoma, the most common site of tumour was the cardia and of the diffuse histologic type (7).

It has been generally accepted that *H. pylori* is linked to the intestinal type of gastric carcinoma while the diffuse and mixed types are due to other environmental influences (3,8). However there has been recent evidence that suggests no difference in occurrence of *H. pylori* between intestinal and diffuse type of carcinoma (3,9,10). In our series the intestinal pattern was the most common histological type with even lower numbers of the diffuse and mixed types.

Conclusion

The frequency of gastric carcinoma is exceptionally low in Kelantan. The Chinese were over-represented. The higher frequency of tumour in the cardia/gastro-oeophageal junction as compared to that of the antrum/body is in sharp contrast to most other series. This adds to the already existing evidence that *H. pylori* is important in the genesis of gastric carcinoma.

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ORIGINAL ARTICLE

PULMONARY TUBERCULOSIS IN HIV INFECTION : THE RELATIONSHIP OF THE RADIOGRAPHIC APPEARANCE TO CD4 T-LYMPHOCYTES COUNT

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Pulmonary tuberculosis (TB) in the AIDS population has a variable chest radiographic presentation. The association between the chest radiographic presentation of pulmonary TB and CD4 T-lymphocyte count in the HIV-infected patient was investigated in order to provide an empirical approach for early diagnosis, treatment, and isolation of these patients. A retrospective analysis of chest radiographs, CD4 T-lymphocyte counts, and clinical history of 80 patients from Hospital Kota Bharu, was performed. All patients were HIV-seropositive and had culture and /or cytology-proven pulmonary tuberculosis. Radiographs were evaluated for the presence of atypical or typical patterns of pulmonary TB. Thirteen (16.2%) patients had typical postprimary pattern, where opacities were distributed at the upper zones, with or without cavitation. Sixty-seven (83.8%) patients had atypical patterns, consisting of normal chest radiograph, middle and/ or lower zones parenchymal opacities, mediastinal lymphadenopathy, pleural effusion and miliary TB. Of these, 18 (22.5%) patients demonstrated normal chest radiographs, 36 (45%) patients showed parenchymal opacities at the middle and/ or lower zones of the lungs, 30 (37.5%) had mediastinal lymphadenopathy, 18 (22.5%) revealed pleural effusion and 6 (7.5%) presented with miliary TB. Sixty-two (77.5%) patients had CD4 T-lymphocytes count less than 200 cells/ul. Of these patients, only 1 (1.6%) had typical pattern. Eighteen (22.5%) patients had CD4 T-lymphocyte count more than 200 cells/ul, where 12 (66.7%) of them showed typical pattern. Patients with CD4 T-lymphocytes count of less than 200 cells/ul, were more likely to produce normal chest radiographs, middle and /or lower zones parenchymal opacities and mediastinal lymphadenopathy. The mean CD4 T-lymphocytes count were also found significantly lower. AIDS patients with pulmonary TB can present with both typical and atypical chest radiograph patterns. An AIDS patient who had CD4 T-lymphocytes count less than 200 cells/ul were more likely to present with atypical radiographic appearance of pulmonary TB. They required appropriate treatment and isolation until the diagnosis of pulmonary TB was confirmed.

Key words : *AIDS; Atypical patterns; CD4 T-lymphocytes count; Chest radiograph; HIV infection; Pulmonary tuberculosis*

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Introduction

The pandemic of HIV infection has had a profound impact on the global TB problem. Due to its ability to destroy the immune system, HIV has emerged as the most significant risk factor for progression of dormant TB infection to clinical disease (1). As a result, the global TB problem has worsened, and it poses an unprecedented medical, social, and economic threat, especially in developing countries. The Global Programme in AIDS of the World Health Organisation (WHO) estimated that, in 1992, at least 9 to 11 million adults and 1 million children has been infected with HIV world-wide. WHO's Tuberculosis Programme has estimated that about 1.7 billion people are infected with *M. tuberculosis* (2). In 1992, there were more than 4 million persons with both HIV and TB infection world-wide, of which more than 3 million lived in sub-Saharan Africa (3). In recent years, an increase in cases of pulmonary TB in the world has been observed and is partially attributed to the increased increased incidence of TB in AIDS population. It is expected that more patients will be diagnosed in the future as the treatment for pulmonary and neurological complication has increased the survival rate of these patients.

Pulmonary TB may not present with the usual radiographic abnormalities when the patient is coinfected with HIV. Studies have described the unusual manifestation of pulmonary TB in patients with advanced stages of HIV infection (4-6). This is due to the alteration of the cell-mediated immunity, the primary aspect of immunity depressed in HIV-seropositive patients. Many investigators (7-9) had attempted to predict the onset of presentation of opportunistic infection and tuberculosis in HIV-seropositive patients on the basis of CD4 T-lymphocyte count. This correlation study of CD4 T-lymphocyte count and radiographic finding is important for early recognition of the pathogen and early institution of treatment as TB is a potentially hazardous but treatable disease.

The purpose of the study is to provide an objective framework for the evaluation of pulmonary TB in HIV-seropositive or AIDS patients, via assessing the common radiographic appearances and to determine whether CD4 T-lymphocyte count correlates with these radiographic findings.

Methods and Materials

Subjects and Clinical Records

Eighty adult patients (75 male, 5 female, mean age 34.5 year old, and range 21 to 51 years) from Hospital Kota Bharu, with positive serology test results for HIV and positive smear/culture of respiratory tract secretions for *M. tuberculosis*, were studied retrospectively between 1 January 1995 and 31 August 1998. Those patients in whom the clinical, laboratory and radiological findings suggest the coexistance of another associated pathological process were excluded from the study.

The patients were labelled as HIV-seropositive if their ELISA screening tests and Western blot tests were reactive. Sputum samples were sent for microscopic examination using Ziehl-Neelsen's method and cultured with Lowenstein-Jensen or Ogawa medium. CD4 T-lymphocyte count was determined by flow cytometry. They were used to assess the magnitude of injury to the host immune system and to monitor the effectiveness of antiretroviral treatment. A count of 200 cells/ul was chosen as the dividing point between groups according to the Centre of Disease Control revised classification system for HIV infection. The CD4 T-lymphocytes count were obtained at or no more than 1 month prior to clinical presentation.

Radiological Examination

A pretreatment posteroanterior (PA) chest radiographs were evaluated by a consultant radiologist, who was blind to the HIV status and CD4 T-lymphocyte count. All radiographs had been obtained within 48 hours of the sputum cytology/ cultures. Evaluation for the presence and location of pulmonary parenchymal opacities, mediastinal and hilar lymphadenopathy, pleural effusions, cavitation and interstitial nodules were performed.

A postprimary *M. tuberculosis* (typical) pattern was defined as airspace consolidation in the apical or upper zone, or superior segment of lower lobe, with or without cavitation and without lymphadenopathy or pleural effusion. The primary (atypical) pattern included middle and/or lower zones opacities, mediastinal or hilar lymphadenopathy, pleural effusion, miliary TB, or a normal chest radiograph. Although we realized that the superior segment of the lower lobe could not be accurately located by frontal chest radiograph alone, presence of infiltrates above and below the horizontal fissure may strongly suggest lesions in this region.

Data Analysis

The *Student's two-tailed t-test* was used to find out the *mean* CD4 T-lymphocyte count among patients with various radiographic findings. The *chi-square test* was used to evaluate the association between subjects with CD4 T-lymphocyte count of above and below 200 cells/ul and their radiographic features. A "*p*" value of less than 0.05 was required for rejection of the null hypothesis. SPSS Version 9 software was used for entering data and statistical analysis. Microsoft Excel was used for graph and table presentations.

Results

Patient and Clinical Data

Eighty adult patients (75 male, 5 female, mean age 34.5 year old, and range 21 to 51 years) from Hospital Kota Bharu, with HIV infection and documented pulmonary TB qualified for the study. Malay patients comprised the majority, i.e., 64 (80%) of the total population, followed by 9 Chinese patients (11.3%) and 7 Siamese (8.8%) patients.

Appearances of Chest Radiographs

Of the 80 patients, 67 (83.8%) presented with atypical patterns. Eighteen (22.5%) patients demonstrated normal chest radiographs. Thirty six (45%) patients had parenchymal opacities at middle or/and lower zones. Mediastinal lymphadenopathy was present in 30 (37.5%) patients, and pleural effusion was found in 18 (22.5%) patients. Only 6 (7.5%) patients presented with miliary TB. Majority of the patients had combination of more than one chest radiographic findings. Table 1 summarises the variable radiographic appearances of atypical patterns in these patients.

CD4 T-Lymphocyte Count and Radiographic Correlation

The CD4 T-lymphocyte count ranged from the lowest reading of 3 cells/ul to the highest value of 478 cells/ul. Majority of the patients fell into CD4 T-lymphocytes count of less than 200 cells/ul, which indicating advanced stage of HIV infection.

Sixty-two (77.5%) patients had CD4 T-lymphocyte count of less than 200 cells/ul. Of those patients, only 1 (1.6%) had typical postprimary chest radiographic appearance. Eighteen (22.5%) patients had CD4 T-lymphocyte count more than 200 cells/ul. Twelve (66.7%) had typical postprimary patterns of pulmonary TB. The detail is further summarized in Table 2.

Those patients whose CD4 T-lymphocyte

Table 1: Variable radiographic appearances of atypical pattern in AIDS population with pulmonary tuberculosis

	Atypical features of PTB in chest radiographs	**Number of patients**
One finding	Middle/lower zones opacities	6
	Lymphadenopathy only	2
	Pleural effusion only	1
	Miliary TB only	3
Combination of two findings	M/L opacities + adenopathy	17
	M/L opacities + pleural effusion	8
	Adenopathy + pleural effusion	2
	Adenopathy + miliary TB	2
	Pleural effusion + miliary TB	1
Combination of three findings	M/L opacities + adenopathy + pleural effusion	7
Total		**49**

count were less than 200 cells/ul were more likely to produce atypical pattern, middle and/or lower zones opacities, normal chest radiograph and mediastinal lymphadenopathy ($p<0.05$). However, there were *no* significant associations between CD4 T-lymphocytes count and the presence of pleural effusions or miliary TB ($p>0.05$). The mean CD4 T-lymphocytes count of the subjects with atypical and normal chest radiograph were significant lower than those with typical and abnormal chest radiograph. This was also true for subjects who had mediastinal lymphadenopathy and middle and/or lower zones parenchymal opacities. However, for pleural effusion and military TB, there were no significant differences in mean CD4 T-lymphocytes count between subjects with or without those features ($p> 0.05$).

Those patients whose CD4 T-lymphocyte count were less than 200 cells/ul were more likely to produce atypical pattern, middle and/or lower zones opacities, normal chest radiograph and mediastinal lymphadenopathy ($p<0.05$). However, there were *no* significant associations between CD4 T-lymphocytes count and the presence of pleural effusions or miliary TB ($p>0.05$). The mean CD4 T-lymphocytes count of the subjects with atypical and normal chest radiograph were significant lower than those with typical and abnormal chest radiograph. This was also true for subjects who had mediastinal lymphadenopathy and middle and/or lower zones parenchymal opacities. However, for pleural effusion and military TB, there were no significant differences in mean CD4 T-lymphocytes count between subjects with or without those features ($p> 0.05$).

Discussion

The radiographic manifestations of adult pulmonary TB in the HIV-seropositive patients are diverse. Much of the literature in the past two decades has described two general patterns of radiographic presentation of pulmonary TB: (a) typical reactivation or postprimary TB and (b) atypical pattern of adult pulmonary TB thought traditionally to be limited to TB of childhood.

Table 2: Radiographic findings according to the CD4 T-lymphocyte count

CD4 T-Lymphocytes count	Radiographic appearances	Number of patients *
Below 200 cells/ul		
a. Atypical presentation (n=61)	i. middle/lower zones opacities	32
	ii. mediastinal lymphadenopathy	27
	iii. pleural effusion	16
	iv. miliary tuberculosis	6
	v. normal chest radiograph	18
b. Typical presentation (n=1)	Upper zone opacities, with or without cavitation	1
Above 200 cells/ul		
a. Atypical presentation (n=6)	i. middle/lower zone opacities	4
	ii. mediastinal lymphadenopathy	3
	iii. pleural effusion	2
	iv. miliary tuberculosis	0
	v. normal chest radiograph	0
b. Typical presentation (n=12)	Upper zone opacities, with or without cavitation	12

* Total for various radiographic findings sum to greater than the total of 80 subjects in the study because some subjects had more than one radiographic finding.

Several literature reviews show that HIV-seropositive patients have radiographic findings more of atypical form (5-10).

Normal radiographic findings in those patients with AIDS and pulmonary *M. tuberculosis* have been reported (6-12). Thus, a normal chest radiograph does not necessary rule out pulmonary TB in HIV infected patients. A study showed that the incidence of pulmonary TB presenting with normal chest radiograph had increased over a period of 10 years (13). There are many possible reasons for this increasing occurrence. One of a likely explanation is improved detection of early disease. The index of suspicion for infectious disease is very high in those patients with HIV infection/AIDS. Thus TB may be diagnosed at an early stage when the chest radiograph is still normal. In addition, procedures and resources directed at appropriate and timely contact tracing had been strengthened. Another contributing factor may be that within the last 10 to 12 years, two laboratory techniques have improved the sensitivity of mycobacterial cultures, facilitating the detection and isolation of smaller numbers of tubercle bacilli. First, high-speed refrigerated centrifugation of processed specimens has improved the concentration of tuberculous bacilli before culture (14). Second, the introduction of the BACTEC 460 TB System, using a broth medium and carbon-14 growth detection system, had improved sensitivity for the isolation of *M. tuberculosis.* The last possible reason was that some of the results had been due to false-positive cultures for *M. tuberculosis.*

Cavitation and atelectasis are less common in the HIV-seropositive group than in the seronegative group. This is not surprising, as one would expect that cavity formation require an intact delayed-type hypersensitivity response and vigorous lymphocyte reactivity to *M. tuberculosis* antigen. Pleural effusions have been documented in several studies to be significantly associated with HIV infection and had been recorded from 12% to 38% of cases in adults (15,16). This picture is different from that seen in children where pleural effusions appear to be significantly less common in those infected with HIV (16). Lymphadenopathy on chest radiograph has been found occurring at the rate of 25% to 50% in HIV infected adults with TB. This variability may result in part from differing stages of the disease, according to the spectrum of CD4 T-lymphocyte count. An American study found that mediastinal adenopathy was common in patients with CD4 T-lymphocyte count of < 200 cells/ul(17), This was confirmed by another report (18). In contrast to this, found that pleural effusion was more common in HIV-infected patients with > 200 CD4 cells/ul, consistent with the current knowledge of the pathogenesis of tuberculous pleuritis (17), which is thought to represent a vigorous local immune response mediated by pleural fluid CD4 cells that secrete interferon in response to *M. tuberculosis* (19, 20). This means that pleural effusion could be regarded as a marker of early clinical HIV disease. A study from South Africa showed that the miliary TB was 8% in HIV-seropositive patients versus 0% in HIV-seronegative patients with TB (21).

The 'atypical' nature of TB in patients with HIV infection is demonstrated clearly by this study. Because of these atypical features, TB is more difficult to diagnose in the setting of HIV infection and may be easily confused with other opportunistic disorders, which can also occur in AIDS patients. For example, the diffuse pulmonary involvement may be easily mistaken for *Pneumocystis carinii* or bacterial pneumonia. Hilar adenopathy and pleural effusion may be manifestations of Kaposi's sarcoma or lymphoma. Coccidiodomycosis, histoplasmosis and *Mycobacterium avium intracellulare* (MAI) can cause chest roentgenographic abnormalities mimicking TB. However, there are still ways to differentiate pulmonary TB from the rest of the lesions mentioned, whether clinically or radiologically. Fairly accurate diagnosis can be made radiographically in majority of cases of *P. carinii* pneumonia (PCP), bacterial pneumonia, and pulmonary TB in HIV-positive patients at the time of hospitalization (22). In approximately 10 percent of cases, these infections may mimic one another radiographically. In this regard, it should be noted that neither *P. carinii* pneumonia nor generalized lymphadenopathy associated with intrathoracic lymphadenopathy (5-23). Thus, the finding of hilar or mediastinal adenopathy in a patient with AIDS is suggestive of TB or infectious process other than *P. carinii* pneumonia. In the case of malignant lymphoma (especially non-Hodgkin lymphoma) or Kaposi's sarcoma, there is usually extrapulmonary evidence of these diseases. Infection with *Mycobacterium avium* Intracellulare (MAI) is the most common nontuberculous mycobacterial infection in AIDS patients. It is ultimately impossible to avoid exposure to MAI due to its ubiquitous distribution. About 30% of AIDS patients will develop generalized MAI infection (24) at some point in the course of their infection. This disseminated form of MAI infection takes place at

a very late stage of AIDS with long-standing low CD4 T-lymphocyte count. More than half of the patients had CD4< 20 cells/ul at the time of MAI diagnosis. On chest radiographs and CT, no reliable distinguishing features between TB and MAI have been found (25) in AIDS patients. Lymphadenopathy is the leading diagnostic feature in both. In MAI there seems to be more pronounced interstitial reaction and less pleural effusion. In patients with undiagnosed pulmonary disease and HIV infection, acid fast organisms identified in pulmonary specimens should be presumed to be *M. tuberculosis*, and appropriate treatment should be instituted.

Some individuals may argue that the distinction between typical and atypical presentations is arbitrary and not necessary. How does this information help the clinician? In developing nations where HIV infection is prevalent without readily available sero-testing and CD4 T-lymphocytes count, TB presenting 'atypically' could be used to estimate the stage of disease (26). The typical pattern of reactivation TB, fibronodular apical opacities, is quite characteristic of TB, and its occurrence on the chest radiograph of a patient immediately raises the suspicion of TB. Appropriate public health measures, such as isolation of the patient, may be rapidly employed. The atypical pattern—focal alveolar opacities, lymphadenopathy, pleural effusions or normal chest radiograph—does not immediately suggest TB and public health measures may be delayed. Establishing a diagnosis of TB at an early stage is, of course, desirable. The patient benefits from early diagnosis and treatment in terms of outcome and complications and from less time and money lost from employment. The clinician avoids the serious consequences of delayed diagnosis or drug resistance by offering single-drug chemoprophylaxis to a patient with current pulmonary TB. Therefore, in those patients with CD4 T-lymphocytes count below 200 cells/ul and atypical chest radiograph, appropriate health measures should be employed until the diagnosis of TB has been reasonably excluded. In some hospitals in the world, as well as in Malaysia, all HIV-infected patients with any form of pneumonia are isolated until TB is ruled out to lessen the risks of nosocomial transmission. Until better means for rapid diagnosis of TB are available, clinicians need to have a high index of suspicion and a low threshold for initiation of treatment if we are to successfully manage TB in the era of HIV and the 'atypical' chest radiograph. These, combined with the high rate of multidrug resistance, emphasise the fact that we are still a long way from eradicating this ubiquitous and often lethal disease.

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Malaysian Journal of Medical Sciences, Vol. 8, No. 1, Januari 2001 (41-45)

ORIGINAL ARTICLE

COMPARISON BETWEEN THE EFFECT OF SOYBEAN AND GOAT'S MILK ON TUMOR-MARKER ENZYME ACTIVITIES DURING HEPATOCARCINOGENESIS IN RATS

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Milk is a physiological fluid which has high nutritional value and soybean has strong antioxidant characteristics which is believed to inhibit carcinogenesis. The objective of this study was to investigate the effects of administration of soybean and goat's milk on hepatocarcinogenesis in rats (fed with diethylnitrosamine; DEN and acetylaminofluorene; AAF) by determining the activities of plasma gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP). Thirty-six rats from the species Sprague-Dawley were divided into 6 groups : control, DEN/AAF, soybean, DEN/AAF with soybean treatment, goat's milk and DEN/AAF with goat's milk treatment. Soybean and goat's milk administrations were given 5 ml/day. The rats were sacrificed after 8 weeks and the blood was collected. Treatment with DEN/AAF caused an increase in ALP and GGT levels and a decrease in weight significantly ($p<0.05$). ALP and GGT activities decreased significantly after administration of soybean and goat's milk ($p<0.05$). Administration of goat's milk and soybean alone did not cause any changes in the enzyme activities. Comparison between the effect of soybean and goat's milk in reducing the enzyme activities (ALP and GGT) did not give significant values ($p>0.05$). However, a decrease in weight was observed in the rats given soybean as well as goat's milk. The results obtained suggested that soybean and goat's milk may work as anti cancer agents in hepatocarcinogenesis although further studies are required to further elucidate this aspect.

Key words : *soybean, goat's milk and hepatocarcinogenesis*

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Introduction

Milk is a physiological fluid which has high nutritional value as it is naturally rich in energy, proteins, vitamins and minerals. The whey protein component in human milk has been reported to pose antitumor activity(1). One study (2) showed that milk fat contains a number of potential anticarcinogenic components including conjugated linoleic acid, sphingomyelin, butyric acid and ether lipids. Conjugated linoleic acid inhibited proliferation of human malignant melanoma, colorectal, breast and lung cancer cell lines. In animals, it reduced the incidence of chemically induced mouse epidermal tumors, stomach neoplasia in mouse and aberrant crypt foci in the rat colon(1).

Soybean possess several naturally occuring phenolic and flavonoids that have strong antioxidant characteristics and are believed to inhibit carcinogenesis. Much attention has focused upon genistein and daidzein, the predominant isoflavones found in soy(3). Epidemiologic, *in vitro* and laboratory animal studies provide evidence for the hypothesis that phytochemicals in soy products have

anticarcinogenic properties(4,5). In addition, protease inhibitors, the Bowman-Birk inhibitor, inositol hexaphosphate (phytic acid), lignans, phytosterols and saponins found in soy products may also have bioactivities relevant to the inhibition of carcinogenesis(4-7).

γ-glutamyl transpeptidase (GGT, E.C 2.3.2.2) and alkaline phosphatase (ALP, E.C 3.1.3.1) are amongst marker enzymes that have been monitored during carcinogenesis (8,9). GGT is a marker enzyme for liver cancer. ALP, a marker enzyme in the liver function test has been reported to be useful as a marker of neoplastic transformation and in hepatocarcinogenesis(10). The objective of this study was to compare the effects of administration of soybean and goat's milk on ALP and GGT activities in hepatocarcinogenesis induced rats using diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF).

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) (Sigma Chemical Co, USA), 2-acetylaminofluorene (AAF) (Sigma Chemical Co, USA), γ-glutamyl carboxynitroanilide (Sigma Chemical Co, USA), p-nitrophenyl phosphate disodium (Sigma Chemical Co, USA) and all other chemicals and other reagents used were of the highest grade commercially available. A basal diet of rat chow was purchased from Gold Coin Co. Ltd. (Malaysia).

Animal Treatment

Male Sprague-Dawley rats (Animal House, Universiti Putra Malaysia), 7-8 week old, weighing 120-150 g, were used. The rats were housed individually in a well-ventilated room (30^0∞C), maintained on normal or treated rat chow and given water *ad libitum*. The rats were divided into six groups of 5-8 rats each. The control group (group 1) was fed a basal diet for the whole duration of the experiment. The soybean and goat's milk control groups (groups 3 and 5) were fed a basal diet and supplemented either soybean or goat's milk 5 ml/ day for every rat. Groups 2, 4, and 6 were induced with hepatocarcinogen; the rats in group 2 were fed a basal diet, the rats in group 4 and 6 were supplemented either soybean or goat's milk at the doses stated previously.

Induction of Hepatocarcinogenesis

Chemically induced hepatocarcinogenesis was carried out using the method of Solt and Farber(11). The rats were injected intraperitoneally with a single dose of DEN at 200 mg/kg body weight. After a 2-week recovery period, the rats were fed a diet (w/w) containing 0.02% (w/w) AAF for 2 weeks. Soybean and goat's milk supplementation

Table 1. Effects of soybean and goat's milk on the activity of Gamma-glutamyl Transpeptidase and Alkaline Phosphatase in plasma induced cancer rats.

Groups	Plasma GGT (IU/L)	Plasma ALP (IU/L)
1. Control	6.19 ± 0.50	343.80 ± 21.98
2. DEN/AAF	10.15 ± 0.66[a]	490.85 ± 36.50[a]
3. Soybean	7.31 ± 0.65[b]	338.32 ± 17.64[b]
4. DEN/AAF + soybean treatment	8.05 ± 0.45[b]	312.68 ± 28.57[b]
5. Goat's milk	6.95 ± 0.37[b]	292.74 ± 29.97[b]
6. DEN/AAF + goat's milk treatment	8.47 ± 0.26[b]	333.29 ± 37.54[b]

Values are means ± SE

a : $p<0.05$ vs control group

b : $p<0.05$ vs induced cancer groups

in the hepatocarcinogen-treated rats was started at the beginning of the DEN administration. The supplementation was continued for a total of 8 weeks after which the rats were killed.

Determination of Marker Enzyme Activities

Blood was collected from the cardiac puncture. Plasma GGT and ALP activities were determined according to the methods described by Jacobs(12) (with some modifications) and Jahan and Butterworth(13), respectively. Both plasma GGT and ALP activities were expressed as IU/L.

Statistical Analysis

The results obtained were analysed using analysis of variance and student's t test. A value of $p<0.05$ was considered as significant.

Results

The results from Table 1 shows that treatment with DEN/AAF caused an increase in ALP and GGT levels and a decrease in weight significantly ($p<0.05$) (Table 2). ALP and GGT activities decreased significantly in DEN/AAF treated rats after administration of soybean and goat's milk ($p<0.05$). Administration of goat's milk and soybean alone did not cause much changes in the enzyme activities when compared to control values. There were no significant differences of ALP and GGT activities ($p>0.05$) among the two treatments. However, a decrease in weight was also observed in the rats given soybean as well as goat's milk (Table 2).

Discussion

The optimum approach to conquering cancer is prevention. Although human diet contains components which promote cancer, it also contains components which may prevent it (2). Observations from animal studies have shown that milk fat reduced the incidence of chemically induced tumors(14). So far, the determination of enzymes in animals supplemented with soy milk or goat's milk with experimentally induced hepatocarcinogenesis has not been reported. It is implied from the results obtained in the present work that soybean and goat's milk reduced the severity of carcinogenesis, as reflected in the reduction of the plasma tumor marker enzymes activities i.e GGT and ALP(8-10). The body weights were affected by the different treatments. A decrease in weight was also observed in rats given soybean as well as goat's milk alone. This may suggest that soybean and goat's milk can be used to decrease or to maintain body weight.

The dairy cow or goat has the ability to extract potential anticarcinogenic agents such as beta-carotene, beta ionone and gossypol from its feed and transfer them to milk(2). In animal studies comparing the tumorigenic potential of milk fat or butter with linoleic acid rich vegetable oils or margarines showed less tumor development with dairy products(2).

Soy protein isolate has been shown to inhibit the growth of human prostate tumors transplanted in mice(14). The *in vivo* inhibition of cancer

Table 2. Effect of different treatment on body weight gained

Groups	Weight Gained (g)
1. Control	218.33 ± 2.19
2. DEN/AAF	170.00 ± 9.36[a]
3. Soybean	171.25 ± 9.04[a]
4. DEN/AAF + soybean treatment	177.67 ± 3.51[a]
5. Goat's milk	160.80 ± 6.87[a]
6. DEN/AAF + goat's milk treatment	177.67 ± 7.47[a]

Values are means ± SE

a : $p<0.05$ vs control group

incidence or progression of cancer by soy products or pure isoflavone has been reported for gastric cancer(15) leukemia(16), breast cancer(17) and others(18). In contrast, some studies have not found *in vivo* inhibitory effects of soy on tumorigenesis. Mc Intosh et al(19)and Rao(20) reported in their studies that soy-based dietary treatments had tumor-promoting effects. Here we report the effect of the soybean supplementation in chemically induced hepatocarcinogenesis. Comparison between the effect of soybean and goat's milk gives not much difference. The differences of the activities of ALP and GGT in both treatments were not significant. It is interesting to note that the soybean exerted an effect similar to goat's milk.

The mechanism of action proposed for the protective effect of soybean and goat's milk during carcinogenesis is similar for vitamin E or C and other antioxidants. The potential mechanisms include scavenging of free radicals produced in hepatocarcinogenesis. Antioxidants are well known to delay or inhibit oxidation and lipid peroxidation.

Conclusion

In conclusion, soybean and goat's milk supplementation caused a reduction in the severity of carcinogenesis, indicating a protective effect of soybean and goat's milk in hepatocarcinogenesis induced rat. The results obtained suggested that soybean and goat's milk worked effectively as anti cancer agents in hepatocarcinogenesis although further studies are required.

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ORIGINAL ARTICLE

THE EFFECT OF -LIPOIC ACID IN BLOOD LIPID LEVELS AND MALONDIALDEHYDE IN ATHEROSCLEROTIC-INDUCED NEW ZEALAND WHITE RABBIT

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-Lipoic acid (ALA) is a naturally occuring cofactor that serves as an acyl carrier in oxidative decarboxylation of _-keto acids in carbohydrate metabolism. Current findings suggest that _-lipoic acid and its reduced form, dihydrolipoic acid (DHLA) may act as antioxidants and are able to quench free radicals in vitro and in vivo. However, the mechanism underlying the process is still unknown. In this study, atherosclerotic lesions were induced in six groups of adult male NZW rabbits labelled as group K, A, B, C, D, E (n=6) by giving 100g/head/day of 2% cholesterol-rich diet for ten weeks. While group K acted as a control, the rest were supplemented with ALA orally (1.4, 2.8, 4.2, 8.0 and 10mg/kg, respectively). In week ten, venous blood samples drawn from ear lobes were analysed for complete lipid profile and peroxidation index. The results showed a significant reduction of total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) levels in most of the treated groups as compared to the control whereas apo-A levels showed a significant increase in group C and D. However, microsomal lipid peroxidation index, malondialdehyde (MDA) was found to be not significantly different. These findings suggest that -lipoic acid may act as a lipid lowering agent in dose dependent manner in premature stage of atherosclerosis but was unable to inhibit lipid peroxidation processes in matured stage of atherosclerosis in rabbits fed a high cholesterol diet.

Key words : *Lipoic acid, atherosclerosis, lipid profiles, lipid peroxidation, antioxidant*

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Introduction

-Lipoic acid, also known as 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or 6,8-thioctic acid has generated considerable clinical interest as a cellular thiol-replenishing and redox-modulating agent (1). Biologically, -lipoic acid (ALA) functions as a cofactor of oxidative decarboxylation reactions in glucose metabolism to yield energy. It has been used for a long time in the western world to treat complications associated with diabetes (2). To carry out this function, the disulfide group of the lipoic acid dithiolane ring is reduced to its dithiol form, dihydrolipoic acid, DHLA (fig 1). Current findings suggest that ALA not only acts as a cofactor in glucose metabolism, but may also act as an antioxidant in vitro and in vivo. In vitro experiments have shown that both ALA and DHLA are potent scavengers of reactive oxygen species. -lipoic acid quenches singlet oxygen, hydroxyl radical and hypochlorus acid (3) while DHLA scavenges hydroxyl radicals, hypochlorus acid, superoxide anions radicals and peroxyl radicals (4).

The observations of ALA as an antioxidant in vitro and in vivo were previously based on hyperglycaemic ambience (2-5). However, until

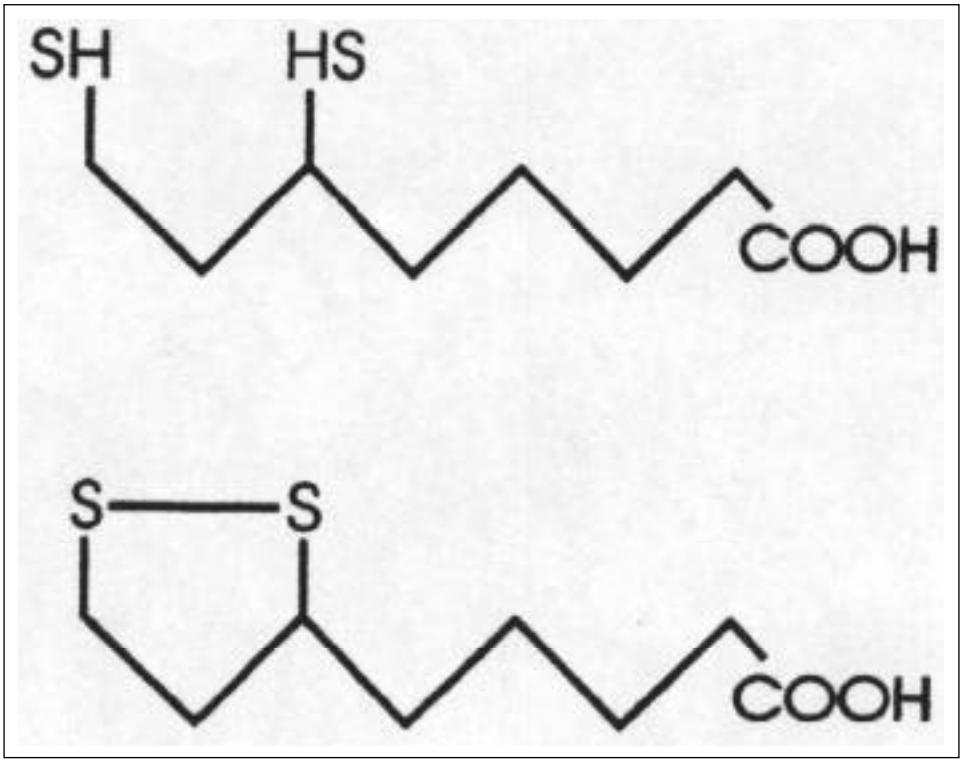
now, no experimental design has addressed the question as to whether -lipoic acid could work in atherosclerotic atmosphere in vivo in preventing proliferation and propagation of this degenerative disease. Lipid peroxidation, the oxidative deterioration of the polyunsaturated fatty acids (PUFA), leads to the formation of hydroperoxides, short-chain aldehydes, ketones and other oxygenated compounds. This process is considered responsible for the development of various diseases like atherosclerosis (6), diabetes (7), cancer (8) and may be one of the main contributing factor towards aging (9). Atherosclerosis, characterised by deposition of cholesterol in and around cells at the intimal layer of the arterial wall is the most important cause of mortality world wide. Although intensive research has been carried out throughout the globe, the precise mechanism of atherogenesis is still uncovered. Hypercholesterolemia most often associated with an elevation of plasma low density lipoprotein-cholesterol (LDL-C) and other related lipids, is believed to be the prime risk factor of atherosclerosis and coronary heart disease (10).

In the present study, we sought to determine whether -lipoic acid was able to exert its antioxidant effect and alter blood lipid levels in atherosclerosis-induced animals.

Materials and Methods

Experimental animals. 36 three month-old male New Zealand White (NZW) rabbits with an average body weight of 2-3 kg were purchased from the Institute for Medical Research Kuala Lumpur (IMRKL). The animals were placed in individual cages and segregated into six groups (n=6) labelled as K (control), A, B, C, D and E. The animals were induced atherosclerotic lesion by giving 100g/head/day of 2% cholesterol-rich diet (ICN Biomedical, USA) for ten weeks. Group A to E were supplemented orally with -lipoic acid (Sigma) at a dose of 1.4, 2.8, 4.2, 8.0 and 10.0mg/kg respectively whereas group K acted as a control. Drinking water was given *ad libitum*. Prior to treatment (day=0) 25 ml of ear lobe venous blood samples were drawn into EDTA venoject tubes placed in a transport ice bucket and were centrifuged at 3000 rpm in a refrigerated bench top centrifuge for 10 minutes at 4 ^{0}C. The plasma samples were analysed for prediet baseline value of TC, LDL-C, HDL-C, TG, apo-A, apo-B and peroxidation index, MDA. In week ten, the animals were sacrificed by exsanguination through common carotid artery after withdrawing the same volume of blood samples from ear vein as previous and the plasma samples

Figure 1: *Dihydrolipoic acid, DHLA (above) and oxidised lipoic acid*



obtained were aliquoted and kept in –70 ^{0} C for a maximum of 7 days before analyses. All experimental procedures in these animals were performed in accordance with protocols approved by the Animal Care & Use Committee, Faculty of Medicine, UKM.

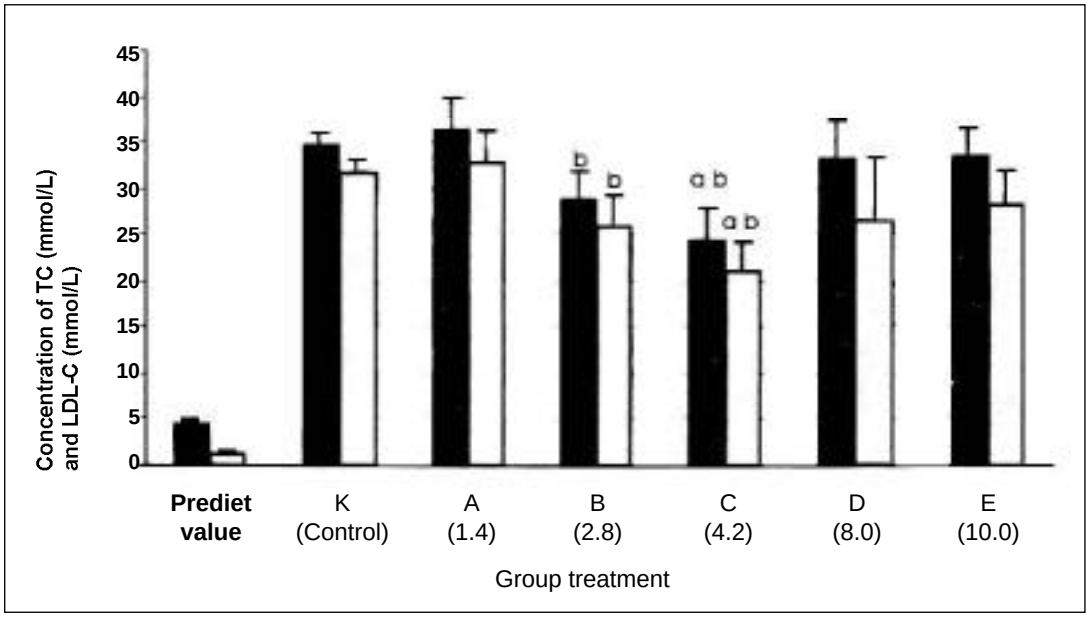
Lipid profiles estimation.

All plasma lipids were estimated using a commercial rapid test kit (Human). Plasma cholesterol was estimated enzaymatically according to principle involving extraction of cholesterol ester by cholesterol esterase(11). The free cholesterol will react with cholesterol oxidase to form cholestenone. The indicator, quinoneimine was formed from hydrogen peroxide, 4-aminoantipyrine and 4-chorophenol under catalytic influence of peroxidase. For this, 0.02 ml plasma was mixed with 2 ml cholesterol reagent, vortexed and incubated at room temperature for 10 minutes. The absorption of pink-coloured quinoneimine was measured at 500nm spectrophotometrically and was multiplied with 5.17 to obtain total cholesterol levels. HDL-C level was estimated by precipitation technique with phosphotungstic acid and magnesium chloride. 0.2 ml of plasma sample was mixed with 0.5 ml HDL pricipitant reagent following incubation at room temperature for 10 minutes. 0.1 ml supernatant obtained after centrifugation for 10 minutes at 4000 g, mixed with 1 ml cholesterol reagent was incubated at room temperature for 10 minutes. The absorbance value measured at 500nm was multiplied with 4.52 to estimate HDL-C level. TG level was estimated by the same method, where 0.01 ml plasma samples was mixed with 1 ml of reagent solution and incubated at 37 ^{0}C for 5 minutes. The absorbance was measured 500nm spectrometrically and was multiplied with 2.28 to obtain TG level. LDL-C level were calculated using Friedwald formula : LDL-C = TC – HDL – [TG/2.2] mmol/L (Friedwald et al. 1972). apo-A and apo-B levels were analysed at 340nm by turbidimetric technique using a commercial kit (Human).

Lipid peroxidation estimation.

Plasma samples obtained were used to study lipid peroxidation in vivo. Malondialdehyde (MDA) as thiobarbituric acid reactive substance was measured at 532nm spectrophotometrically (12) whereas the microsomal protein concentration was determined by Lowry's method (13).

Figure 2: *Level of total cholesterol, TC and low density lipoprotein-cholesterol, LDL-C in high cholesterol diet, pretreatment (far left bars) and post-treatment of various doses of alpha-lipoic acid (ALA). Number in the bracket below the group label indicated the dose (mg/kg). $p < 0.05$ considered statistically significant. $^a p < 0.05$ from control, $^b p < 0.05$ from group A. Prediet bars provided is for comparison purposes.*



Statistical analysis**.**

All data were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA and $p<0.05$ were considered significant. Tukey post-tests were performed for multiple group comparison.

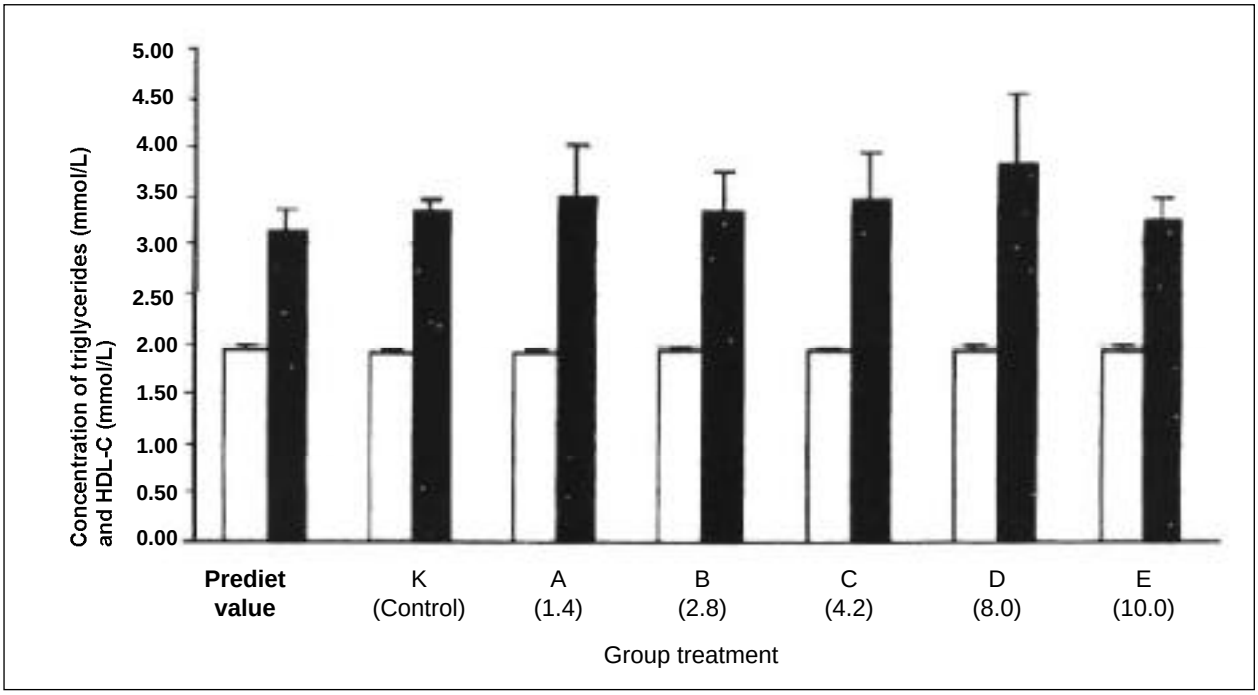
Results

Prediet baseline value for lipids and lipoproteins in NZW rabbits at the start of the study were 4.63 ± 0.15 mmol/L for TC, 3.15 ± 0.22 mmol/L for TG, 1.92 ± 0.08 mmol/L for HDL-C, 1.28 ± 0.18 mmol/L for LDL-C, 19.46 ± 5.09 mg/dL for apo-A, 4.72 ± 0.91 mg/dL for apo-B whereas MDA level was 0.04 ± 0.01 nmol/mg protein. At the end of week ten, venous blood were once again withdrawn and analysed as appropriate. The analysis revealed that the level of TC was significantly reduced in most of the treatment groups compared to control, with group C (4.2 mg/kg of ALA treatment) exhibiting the lowerst TC level (24.45 ± 3.34 mmol/L) followed by group B and A (29.02 ± 3.22 mmol/L and 36.32 ± 3.37 mmol/L, respectively) (fig.2). In group D and E, post-treatment TC levels however increased. The levels of low density lipoprotein cholesterol (LDL-C) were observed to reduce significantly in a similar pattern to that of TC levels with group C showing the lowerst cencentration (20.94 ± 3.48 mg/dL). Similar to TC level, LDL-C level increased in both group D and E. Neither TG nor HDL-C gave a significant difference between control and treatment group (fig. 3). For lipoprotein profiles, apo-A levels were increased significantly in group D (54.99 ± 9.61 mg/dL) followed with group C (40.08 ± 9.30 mg/dL) (fig 4), whereas apo-B did not show any significant difference. Plasma lipid peroxidation index, MDA also did not show a significant difference in any of the treated groups (fig.5).

Discussions

As the world rapidly progresses, the society becomes more affluent with the lifestyle changes. High consuming power has led to the people comsuming more food rich in fat, sugar, salt and cholesterol. Sedentary lifestyle, increased stress coupled with environmental pollution and the changing lifestyle have significantly contributed towards increased incidence of atherosclerotic-related diseases. These days however, more and more people are now beginning to look upon natural products or naturopath (14) as an alternative supplement in preventing diseases. -lipoic acid

Figure 3: *Level of triglycerides, TG and high density lipoprotein-cholesterol, HDL-C in high cholesterol diet, pretreatment (far left bars) and post-treatment of various doses of alpha-lipoic acid (ALA). Non of these parameter reveals a significant different between prediet (day=0) values or post-treatment.*



extracted in 1950s and being realised by scientist to have an antioxidant effect in the last decade, may play a significant role in disease prevention measures. -lipoic acid is a fat and water-soluble, sulphur containing coenzyme which is involved in energy production. A related metabolic functions of -lipoic acid is its role in blood glucose disposal (2) through the glucose-metabolizing enzymes, pyruvate dehydrogenase and alpha-ketogluturate dehydrogenase eventhough some researchers suspects a more direct role in cellular glucose uptake at the cellular membrane (15). However, the involvement of this substance in cardiovacular related diseases have not been studied. Current findings suggest that pathogenesis of atherosclerosis involves oxidation of LDL-C particles via free radical activity (5-9). Elevated concentration of LDL-C in the circulation may further contribute the so-called bad cholesterol to penetrate into the vascular wall followed by macrophage activation to form foam cell. Cellular interaction was believed to be involved in propagating advanced-stage atherosclerosis lesion particularly MCP-1 and M-CSF molecule which play a role in macrophage recruitment and migration whereas cytokins such as IL-1 and PDGF contribute towards SMC proliferation at the site of lesion (17).

Previous studies noted that ALA through its reduced form DHLA exhibits a protective effect against free radical activity eventhough its precise mechanism is still not understood. In our present study, MDA level was not significantly different in the treated group compared to the control. This seems to be in contrast to previous findings that suggest ALA would be a potent metabolic antioxidant to quench free radicals in vitro and in vivo (9,17-18). For the plasma lipid analysis, a significant reduction of TC and LDL-C were observed. This data may suggest a new lipoate activity in vivo. Both TC and LDL-C levels were maximally reduced by 4.2mg/kg of ALA supplementation. The reduced levels however were still high compared to the prediet baseline value. Following 8.0 and 10.0mg/kg, these lipid levels increased. These results suggest that ALA supplementation may increase lipid and lipoprotein regulation. The mechanisme of how ALA is able to reduce LDL-C and TC concentration is unknown.

Figure 4: *Level of apo-A and apo-B in high cholesterol diet, pretreatment (far left bars) and post-treatment of various doses of alpha-lipoic acid (ALA). Supplementation 4.2 mg/kg and 8.0 mg/kg of ALA gives a significant increase in apo-A concentration indicating ALA capable to enhance apo-A protein synthesis probably through the hepatic system although matured form HDL-C do not simultenously increase. apo-B reduces accordingly but do not achieved statistically significant. $p < 0.05$ was considered statistically significant; $^{a}p < 0.05$ significant from control, ; $^{b}p < 0.05$ significant from group A, ; $^{c}p < 0.05$ significant from group B, ; $^{d}p < 0.05$ significant from group C.*

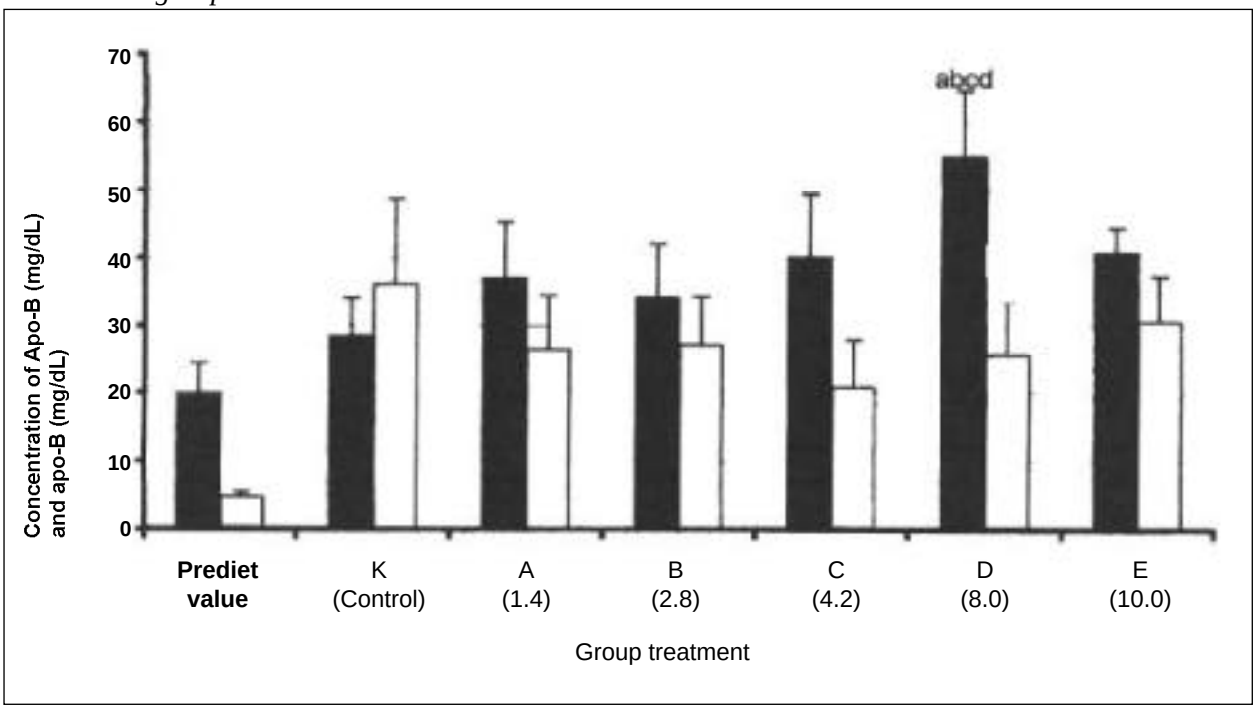
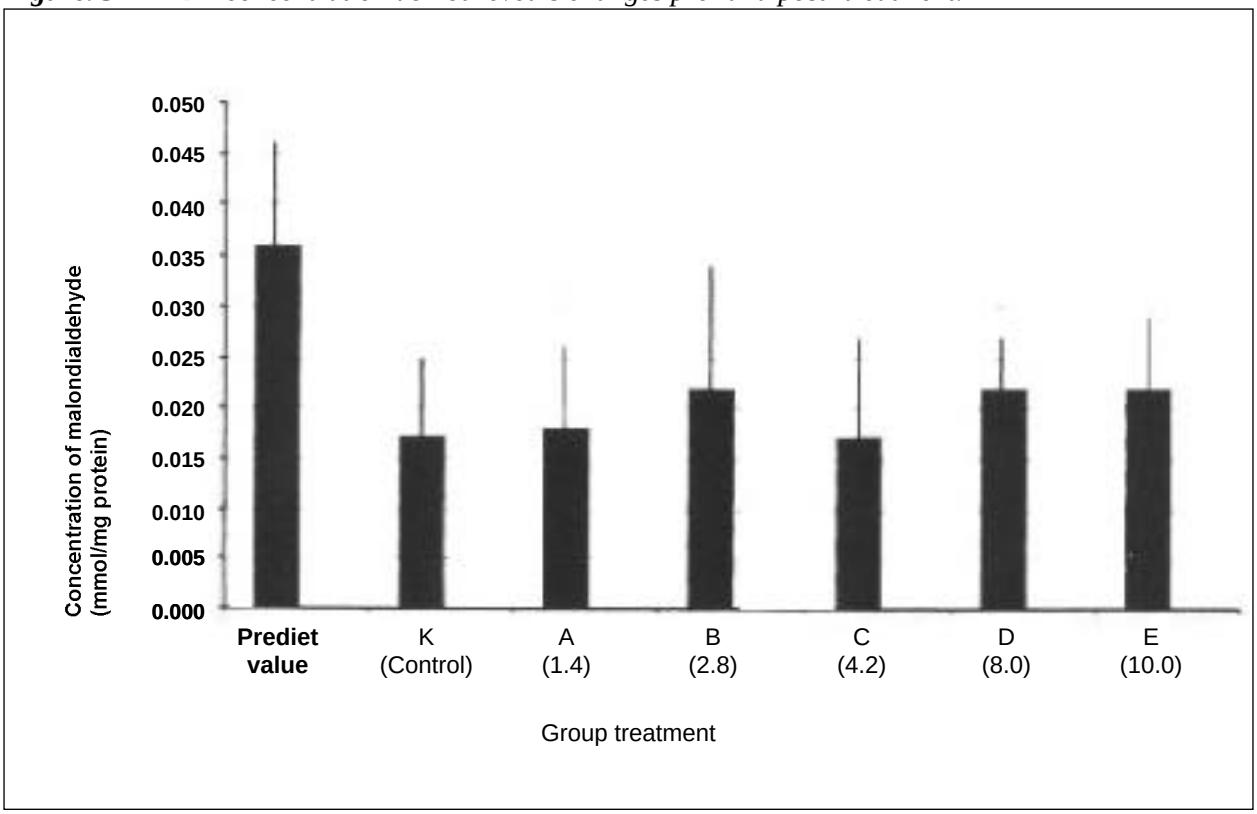


Figure. 5 *MDA concentration do not reveals changes pre- and post- treatment.*



Probably it may be via lipoprotein lipase (LPL) activity or through cholesterol metabolism by the liver. Chiba et al. reported increased LPL activity and HDL-C level in cholesterol fed NZW rabbit after administration of NO-1886 (19). ALA probably initiates LDL receptor synthesis in the liver which in turn increases the uptake of cholesterol back to the hepatic system and increased synthesis of apoprotein A for reverse cholesterol transport (20). However, at higher doses, a reverse effect may occur (18). These data may provide information that beyond the optimum dose, ALA could be toxic to living tissue. However, the treshold concentration for this molecule to exhibit its optimum effect needs furthur investigations. This study also reveals that prolonged intake of high cholesterol diet may cause retention of LDL-C and TC even with supplementation of ALA. Reduction of LDL-C and TC would probably depend on the cholesterol concentration in the diet. More and more workers have now started using diets with less than 2 % cholesterol for induction of atherosclerosis. With consideration to the dose of ALA, progression of atherosclerotic disease probably could be reduced to some extent. Although the present data do not allow the conclusion that ALA supplementation could prevent athrosclerosis related free radical activity, the data are in agreement with a model in which ALA supplementation at an optimum dose may contribute to reduction of bad cholesterol in the circulatory system. (21-24)

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ORIGINAL ARTICLE

DETECTION OF VANCOMYCIN-RESISTANT *ENTEROCOCCUS* SPP. (VRE) FROM POULTRY

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Twenty-eight isolates of *E. faecalis* and 5 isolates of *E. hirae* were isolated from chicken samples obtained from markets in Sri Serdang, Selangor. They were tested for susceptibility to vancomycin and other antimicrobial agents. All of the isolates showed multiple resistance to the antibiotic tested. All *Enterococcus* spp. were resistant (100%) to ceftaxidime, cephalothin, erythromycin, gentamicin, kanamycin, nalidixic acid and streptomycin. Resistance was also observed to norfloxacin (97%), tetracycline (91%), penicillin (85%), bacitracin (82%), chloramphenicol (61%) and the least resistance was to ampicillin (27%). High prevalence to vancomycin resistance was detected among the *E. faecalis* (27of 28) and *E. hirae* (4 of 5) isolates. The multiple antibiotic resistance index ranging between 0.64 to 1.0 showed that all strains tested originated from high-risk contamination. Plasmid profile analysis of *Enterococcus* spp. revealed plasmid DNA bands ranging in size from 1.3 to 35.8 megadalton but some isolates were plasmidless. No correlation could be made between plasmid patterns and antibiotic resistance.

Key words : *Enterococcus spp., vancomycin-resistant, plasmid, poultry*

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Introduction

Enterococcus species usually inhabit the intestines of human and other animals. These organisms were considered as a part of normal flora of the bowel, genital tract with some also being found on the skin, vaginal secretions and in the perineal area. The genus *Enterococcus* are gram-positive cocci that are catalase negative, occur singly, in pair and short chains.

Enterococci have been increasingly involved in nosocomial infections, sometimes as a cause of hospital outbreaks (1). In recent years, they have emerged as pathogens in a growing number of serious nosocomial and urinary tract infections including bacteremia and intraabdominal (2,3). The isolation of strains resistant to many antibiotic therapies has become an important public health concern (4,5,6). Once, the glycopeptide antibiotic agent, vancomycin was useful in the treatment of severe infections due to gram-positive bacteria (7,8). Unfortunately, resistance to vancomycin had been reported (9).

Our aim was to isolate and investigate the resistance of enterococcal poultry isolates to various antimicrobial agents including glycopeptide (vancomycin) as well as to determine their plasmid profiles.

Materials and Methods

Isolation of Vancomycin-Resistant Enterococci (VRE)

All thirty-three isolates of Enterococci were isolated from chicken meat samples (chicken breasts, chicken legs and other chicken parts) obtained from markets in Sri Serdang, Selangor.

Approximately 25 g of each poultry product was rinsed in 225 ml of azide dextrose broth and homogenized with a stomacher for 1 min. After overnight incubation at 37^0C, 0.1 ml of the diluted sample was plated on Slanetz and Bartley agar (SBA) supplemented with 20 µg/ml of vancomycin. The agar plates were incubated aerobically at 37^0C for 24 hour. Typical red colonies from each SBA plate were randomly isolated and investigated further.

Species Identification

Presumptive identifications of the *Enterococcus* spp. were performed by using the following characteristics: Gram stained reaction, colony morphology, growth and blackening of bile-esculin agar, growth in the presence of 6.5% NaCl and growth at 10^0C and 45^0C, the presence or absence of catalase and acidification of glucose with the production of gas (10).

Identification of the strains was further investigated to the genus level by growth and biochemical reactions as described by Facklam and Collins (11).

Plasmid isolation

The plasmid DNA of *Enterococcus* spp. strains were screened by the alkaline lysis method of Birnboim and Doly (12) with slight modification. The products were then electrophoresed for 1 hour at 150V on a 0.8% agarose gel. After staining the gel with ethidium bromide (0.5 µg/ml), the photograph was taken. Molecular mass of the plasmid was determined by approximate comparison with plasmid of known molecular weight, *E.coli* V517 that harboured 8 plasmid of 1.4 to 35.8 MDa (13).

Antimicrobial Susceptibility Testing

Table 1: *Frequency of antibiotic resistance of the thirty-three* Enterococcus *spp. tested*

Antibiotic	No. (%) of resistant strains		
	E. faecalis (28 strains)	E. hirae (5 strains)	Total (33 strains)
Ampicillin	7 (25)	2 (40)	9 (27)
Bacitracin	25 (89)	2 (40)	27 (82)
Chloramphenicol	18 (64)	2 (40)	20 (61)
Ceftazidime	28 (100)	5 (100)	33 (100)
Cephalothin	24 (86)	5 (100)	33 (100)
Erythromycin	28 (100)	5 (100)	33 (100)
Gentamicin	27 (96)	5 (100)	32 (97)
Kanamycin	28 (100)	5 (100)	33 (100)
Nalidixic acid	28 (100)	5 (100)	33 (100)
Norfloxacin	27 (96)	5 (100)	32 (97)
Penicillin	24(86)	4 (80)	28 (85)
Streptomycin	28 (100)	5 (100)	33 (100)
Tetracycline	25 (50)	5 (100)	30 (91)
Vancomycin	27 (96)	4 (80)	31(94)

***Table II**: Plasmid profiles and antibiotic resistance patterns of* Enterococcus *spp. isolates*

Strains	Antibiotic resistance[ab]	MAR Index	Plasmid profiles (MDa)[c]
HTF101	BCCazCfEGmKNaNorPSTeVa (1)	0.93	35.8 (1)
HTF102	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	35.8, 2.9 (2)
HTF103	BCCazCfEGmKNaNorPSTeVa (1)	0.93	35.8, 1.9, 1.3 (3)
HTF104	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	35.8, 1.9, 1.3 (3)
HTF105	BCCazCfEGmKNaNorPSTeVa (1)	0.93	3.8 (4)
HTF106	BCCazCfEGmKNaNorPSTeVa (1)	0.93	35.8 (1)
HTF107	BCCazCfEGmKNaNorPSTeVa (1)	0.93	-[d]
HTF108	BCCazCfEGmKNaNorPSTeVa (1)	0.93	-
HTF109	BCazCfEGmKNaNorPSTeVa (3)	0.86	35.8, 5.8 (5)
HTF110	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	5.0 (6)
HTF111	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	1.9, 1.3 (7)
HTF112	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	35.8, 1.9, 1.3 (3)
HTF113	AmBCazEGmKNaNorPSTeVa (4)	0.86	-
HTF114	BCCazCfEGmKNaNorPSTe (5)	0.86	35.8 (1)
HTF115	CCazEGmKNaNorSTeVa (6)	0.71	-
HTF116	CCazCfEGmKNaNorPSTeVa (7)	0.86	-
HTF117	AmBCCazCfEGmKNaNorPSTeVa (2)	1.0	-
HTF118	BCCazCfEGmKNaNorPSTeVa(1)	0.93	-
HTF119	BCCazCfEGmKNaNorPSTeVa(1)	0.93	-
HTF120	BCazEGmKNaNorPSTeVa(8)	0.79	-
HTF121	BCazCfEKNaNorPSVa(9)	0.71	-
HTF122	BCazCfEGmKNaNorPSTeVa(3)	0.86	-
HTF123	BCCazCfEGmKNaNorPSTeVa(1)	0.93	-
HTF124	BCazCfEGmKNaSTeVa(9)	0.71	-
HTF125	BCazCfEGmKNaNorSTeVa(11)	0.79	-
HTF126	BCazEGmKNaNorPSTeVa(8)	0.79	-
HTF127	CazCfEGmKNaNorPSVa (12)	0.71	-
HTF128	BCazCfEGmKNaNorSTeVa (11)	0.79	-
HTH101	CazCfEGmKNaNorSTe (13)	0.64	-
HTH102	AmCazCfEGmKNaNorPSTeVa (14)	0.86	6.0, 3.7 (8)
HTH103	AmBCazCfEGmKNaNorPSTeVa (15)	0.93	7.0, 3.7, 2.8 (9)
HTH104	BCCazCfEGmKNaNorPSTeVa (1)	0.93	7.0, 3.7, 2.8 (9)
HTH105	CCazCfEGmKNaNorPSTeVa (7)	0.86	1.8, 1.5 (10)

[a]Tested for ampicillin (Am), bacitracin (B), chloramphenicol (C), ceftazidime (Caz), cephalothin (Cf) erythromycin (E), gentamicin (Gm), kanamycin (K), nalidixic acid (Na), norfloxacin (Nor), penicillin (P), streptomycin (S), tetracycline (Te), vancomycin (Va)

[b,c] Number in parenthesis indicates antibiotypes group and plasmid patterns group

[d]None detected

All isolates identified as enterococci were tested by disk diffusion tests on tryptic soy agar (11). All strains were tested for their susceptibility to ampicillin at 10 µg, bacitracin at 10 µg, chloramphenicol at 30 µg, ceftazidime at 30 µg, cephalothin at 30 µg, erythromycin at 15 µg, gentamicin at 10 µg, kanamycin at 30 µg, nalidixic acid at 30 µg, norfloxacin at 30 µg, penicillin at 10 U, streptomycin at 10 µg, tetracycline at 30 µg and vancomycin at 30 µg.

The multiple antibiotic resistance (MAR) index of isolates was defined as a/b where 'a' was the number of antibiotics to which the isolate was resistance and 'b' was the total number of antibiotics tested (14).

Results

From the 33 vancomycin-resistant Enterococci (VRE) isolated from the chicken meat examined, 28 (85%) strains were identified as *E. faecalis* and 5 (15%) as *E. hirae*. Generally the characteristics of the isolates agreed with previous studies that the genus *Enterococcus* comprised of gram-positive cocci that are catalase negative, grow in 6.5% NaCl and at pH 9.6. They grow both at 10^0C and 45^0C and none produced gas from glucose. They also grew on and blackened 40% bile-esculin agar (11, 15, 16). All thirty-three isolates of Enterococci were resistant to 9 or more antibiotic tested. The highest prevalence of resistance observed among the isolates were against ceftazidime, cephalothin, erythromycin, gentamicin, kanamycin, nalidixic acid and streptomycin (100%). The least resistance was observed for ampicillin (27%). Table I showed the resistant pattern among the *Enterococcus* spp. tested. The results of plasmid profile analysis among the *Enterococcus* spp. isolates were shown in Table II. Fifteen of the isolates harbour one or more plasmid DNA bands ranging in sizes from 1.3 to 35.8 megadalton.

Discussion

The isolation of vancomycin-resistant enterococci (VRE) species in this study was to investigate the importance of chicken meat as a possible source for the transfer of VRE. The results obtained showed the presence of VRE in the chicken samples examined and similar reports have been published on the occurrence of *Enterococcus* spp. from animal sources (17, 18, 19, 20, 21).

There is little information on the resistance to antibiotics among *Enterococcus* spp. in Malaysia. In this study, 94% of the isolates were vancomycin-resistant and more than 50% of the Enterococci isolates acquired high-level resistance to other antibiotics tested, reflecting the distribution of aminoglycoside resistance worldwide and resistance to other antibiotics among VRE worldwide (22). The resistance patterns in this study generally agreed with the observations reported by Murray (1) and Son *et al.* (23) on the prevalence of multiple drug-resistant enterococci. Twenty-seven of 28 *E. faecalis* and 4 of 5 *E. hirae* were resistant to vancomycin, which indicated the high prevalence of vancomycin-resistant enterococci from chicken samples tested in the study area. In addition, all isolates of *Enterococcus* spp. from poultry sources used in this study had multiple antibiotic resistance (MAR) indices of 0.64 to 1.0, indicating that all strains originated from high-risk sources (14). Elsewhere, a frequent occurrence of antimicrobial resistance enterococci has been observed among food animals and food of animal origin (17, 19, 21, 24, 25). Taken together, the results of this study and those cited above suggested that food animals might be a reservoir of resistant enterococci and resistance gene capable of transferring to human through the food chain.

The results of the plasmid screening generally agreed with previous studies by Son *et al.* (23) who reported the occurrence of small and large plasmid DNA compared to Boyce *et al*. (26) who revealed that all isolates of VRE examined contained a common 40 MDa plasmid. Taken together, these results suggest that plasmids in VRE are of variable size. Bacterial plasmid are known to confer a variety of phenotypic modifications and genetic flexibility upon their host by carrying genes that may code for toxin production and antibiotic resistance (27). Plasmid screening by agarose gel electrophoresis revealed 10 plasmid profiles scattered in 33 of the isolates. Fifteen antibiotypes were identified among 33 *Enterococcus* spp. strains based on the evidence of resistance patterns. Though vancomycin resistant among clinically important Gram-positive species had not been widely reported before 1986, over the last decade has witness the emergence of glycopeptide resistance from negligible rate to clinically problematic levels (28, 29, 30). Glycopeptide resistance in enterococci is thought to be principally plasmid-mediated and the ability to transfer resistant genetic material among Gram-posiotive strains and species has been demonstrated (29, 30, 31, 32). This renders *Enterococcus* species

that have been previously considered of minor clinical importance, significant if associated with either multiple resistance factors or as a reservoir of resistance genes as observed in this study. However, at this stage of this study no specific correlation between the antibiotic patterns and plasmid profiles was observed. Further evidence on the correlation of the presence of plasmids and antibiotic resistance could be obtained by conjugation, transformation or curing experiments.

In conclusion, our findings showed that multiple resistant VRE isolates are already present in poultry and thus, there is every reason to be concerned as human infection due to VRE may stem from poultry sources.

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ORIGINAL ARTICLE

PLASMID-MEDIATED STREPTOMYCIN RESISTANCE OF *LISTERIA MONOCYTOGENES*

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A strain of streptomycin-resistant *Listeria monocytogenes* LM35 isolated from imported frozen beef was examined in this study. In conjugation studies, the *L. monocytogenes* LM35 strain harbouring two plasmids of 54, 3.0, 2.8 and 2.7 kilobase was used as the donor and streptomycin-sensitive and plasmidless *L. monocytogenes* LM65 and LM100 strains as the recipients. Streptomycin resistance was transferred to *L. monocytogenes* LM65 and LM100 strains at frequencies of 3.3×10^{-8} and 1.2×10^{-9} per input donor cells, respectively. In both occasions, we also observed the concomitant transfer of the donor's 54 kilobase plasmid. These results suggest that streptomycin resistance in *L. monocytogenes* LM35 was mediated by the 54 kilobase plasmid.

Key words : *Listeria monocytogenes*, plasmid, streptomycin, transfer

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Introduction

Listeria monocytogenes is a gram-positive opportunistic pathogen responsible for severe infections (septicemia, meningitis and meningoencephalitis) primarily in immunocompromised hosts, the elderly, neonates, and foetuses. Infections caused by *L. monocytogenes* are likely to be foodborne as the intestinal tract is the most probable site of invasion (1). *Listeria* contamination occurs in a wide range of foods such as dairy products, vegetables, raw fish, fermented sausage, meat and poultry (2-5). In Malaysia, beef is a popular food. Multi-antibiotic resistance plasmids encoding resistance to chloramphenicol, macrolide/lincosamide/streptogramin, tetracycline, erythromycin and streptomycin have been found in *Listeria monocytogenes* (6-7). To the best of our knowledge, there has been no report yet on plasmid-mediated antibiotic resistance among *Listeria monocytogenes* from food sources in Malaysia. In our previous study, we reported on the conjugative transfer of plasmid-mediated kanamycin resistance in *Listeria innocua* strain isolated from fermented fish (8). Thus, there is a need to assess the transferability of antimicrobial resistance of *Listeria monocytogenes* to establish the possible hazards to public health due to digestion of foodborne resistant strains.

The objective of the present study was to determine whether genetic information coding for streptomycin resistance in *L. monocytogenes* strain LM35 may be carried on conjugative R plasmid.

Materials and Methods

Bacterial conjugation

A streptomycin-resistant *Listeria monocytogenes* LM35 harbouring a 54 kilobase plasmid and three small plasmids of 2.7, 2.8 and 3.0 kilobase in sizes (donor strain, see Figure 1), and streptomycin-sensitive and plasmidless *L. monocytogenes* LM65 and LM100 (recipient strains)

isolated from imported frozen beef used in this study have been described previously (9).

Donor and recipient cells were grown to mid-log phase (10^7 cfu/ml) in tryptic soy broth (TSB) at 35°C. A 0.5 ml sample of the donor strains was added to 1.0 ml of the recipient sample on a tryptic soy agar (TSA) plate, and incubated overnight at 35°C. Bacteria were harvested from the TSA plate and a ten-fold serial dilutions of each mating mixtures in saline (0.85%) were spread on plates supplemented with 30 µg of streptomycin and tetracycline (*L. monocytogenes* LM65) or streptomycin and chloramphenicol (*L. monocytogenes* LM100) to which the recipients were resistant, respectively. Plate counts were performed for estimates of donor and recipient population on TSA plates containing antibiotic to which the donor or recipient strains were resistant, respectively. Colonies growing on this double-inhibitor-supplemented medium after 24 to 48 h of incubation at 35°C were scored as presumptive transconjugants, and the frequency of transfer was calculated as the number of transconjugants per initial number of donors. Ten or more transconjugants from each mating were picked and tested for their antibiotic resistance as described previously (8).

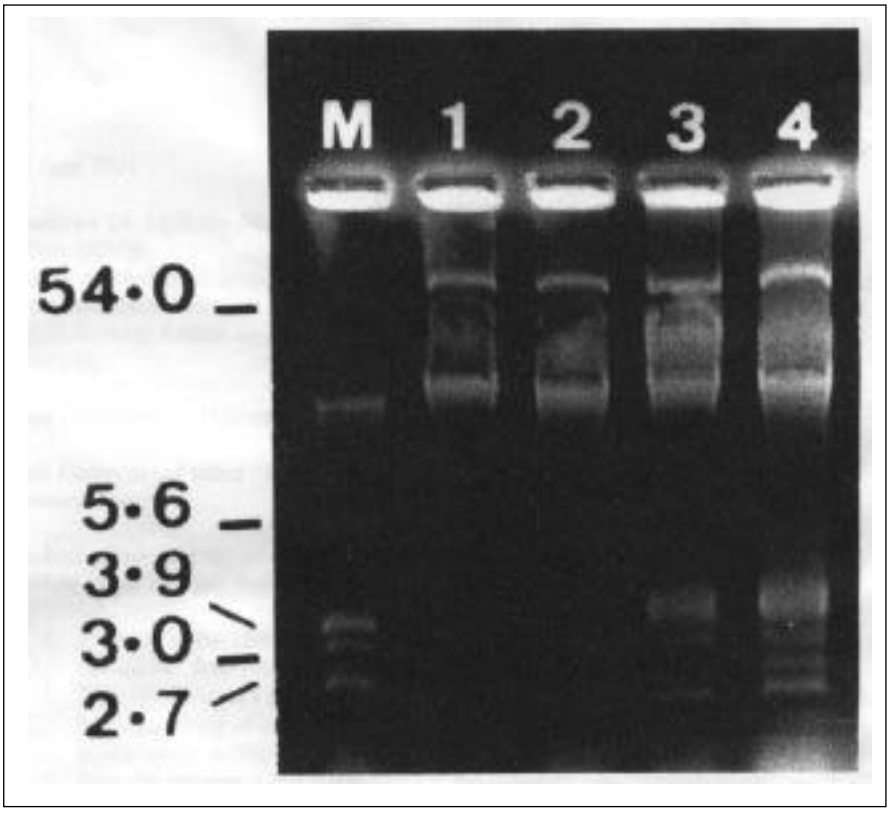
Plasmid isolation

Streptomycin-resistant transconjugants were screened for the presence of plasmid by the method of LeBlanc and Lee (10). Extracted plasmids were electrophoresed for 2 h at 35 mA on a 0.85% agarose gel in TBE buffer (89 mM Tris-base - 89 mM boric acid - 2.5 mM disodium RDTA) as described by Sambrook *et al*. (11). The approximate molecular mass of each plasmid was determined by comparison with plasmid of known molecular mass from *E. coli* V517 (12).

Results and discussion

The presence of streptomycin resistance in *Listeria monocytogenes* LM35 prompted us to investigate whether this strain could act as donor of streptomycin resistant in mating experiments with the streptomycin-sensitive *Listeria monocytogenes* LM65 and LM100 strains. In three independent experiments the *L. monocytogenes* LM35 strain was able to transfer streptomycin resistance to recipient

Figure 1: Agarose gel (0.85%) gel electrophoresis of plasmid DNA from *L. monocytogenes* strains and their respective transconjugants. Lanes: 1, transconjugant LM65; 2, transconjugant LM100; 3 and 4, donor L35; M, E.



listeriae. The frequencies of transfer, expressed as the number of transconjugants per donor colony forming unit (CFU), were 3.3 x 10^{-8} to *L. monocytogenes* LM65 and 1.2 x 10^{-9} to *L. monocytogenes* LM100. The 54 kilobase plasmid of the donor was detected in the streptomycin-resistant transconjugants (Figure 1). However, the 2.7, 2.8 and 3.0 kilobase plasmids were not transferred to the streptomycin-resistant transconjugants. Antibiotic resistance is often determined by genetic information of plasmid origin and that the correlation between antibiotic resistance and plasmid profile may indicate that the genetic information is plasmid-borne (13). Thus, it was apparent from the results obtained in this study that the streptomycin resistance phenotype of the *L. monocytogenes* LM35 strain was mediated by the 54 kilobase plasmid. However, it should be noted here that further evidence to support the finding on the conjugal transfer of the streptomycin resistance and the 54 kilobase plasmid can be obtain by conducting curing and hybridization experiments or cloning of the streptomycin resistance gene from the 54 kilobase plasmid.

On the basis of their studies of antibiotic resistance in *L. monocytogenes*, and more particularly the transferability of streptomycin resistant between *L. monocytogenes* and *E. faecalis*, Poyart-Salmeron *et al*. (14) suggested that enterococci might be a reservoir of resistance for *L. monocytogenes*. However, since the *L. monocytogenes* LM35 strain examined in this study harboured a self-transmissible plasmid, our results may suggest that *L. monocytogenes* could act as a reservoir of streptomycin resistance genes for intra- and intergeneric dissemination of antibiotic resistance. Transfer of resistance between *L. monocytogenes* and other bacterial species might occur in the gastrointestinal tract of domestic animals and man where these species may live. Since the intestinal tract represents the portal entry for *Listeria* strains (14-16) human infections caused by antibiotic-resistant *L. monocytogenes* from food may occur.

In conclusion, since listeriosis is often fatal even with antibiotic therapy, there is every reason to be concerned at reports of *Listeria monocytogenes* with plasmid-mediated antibiotic resistance as evidenced by the results obtained in this study. Thus food samples may need to be monitored for the emergence of resistant strains.

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ORIGINAL ARTICLE

BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FERMENTED FOOD

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Lactic Acid Bacteria (LAB) isolated from several traditional fermented foods such as "tempeh", "tempoyak" and "tapai" were screened for the production of bacteriocin. One strain isolated from "tempeh" gives an inhibitory activity against several LAB. The strain was later identified as *Lactobacillus plantarum BS2*. Study shows that the inhibitory activity was not caused by hydrogen peroxide, organic acids or bacteriophage. The bacteriocin production was maximum after 10 hours of incubation with an activity of 200 AU/ml. The bacteriocin was found to be sensitive towards trypsin, α-chymotrypsin, β-chymotrypsin, α-amylase and lysozyme.

Key words : *Lactic acid bacteria, bacteriocin,* Lactobacillus plantarum

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Lactic acid bacteria is an important group of industrial microorganism involved in the processing of various fermented food which include vegetables and sausages, dietary adjuncts, probiotics and even cosmetic ingredients (1). It is used as starter culture to improve the texture and the flavour of the products. The ability to inhibit growth of spoilage microorganisms and pathogenic bacteria contribute to the maintenance of hygienic and quality of the products or host health. This inhibitory activity is the result of the metabolic product secreted by these LAB which acts as antimicrobial compounds. These compounds include organic acids, diacetyl, hydrogen peroxide and bacteriocin (2).

Recently, there has been much interest in bacteriocin synthesized by LAB. Bacteriocins, are defined as bioactive peptides or protein with an antimicrobial activity towards gram positive bacteria including closely related species and/or food spoilage and pathogenic bacteria such as *Bacillus cereus, Clostridium botulinum, Staphylococcus aureus* and *Listeria monocytogenes* (3). The use of bacteriocin or bacteriocin producing culture as potential 'biopreservatives', and possibly for replacing chemical preservatives (4) has received much attention. This is due to current awareness of consumers towards the use of food preservatives. The purpose of this study is to screen lactic acid bacteria isolated from Malaysian traditional fermented food such as "tempeh", "tapai" and "tempoyak" for their ability to produce bacteriocin which exhibit antagonistic activity against indicator strains.

Materials and methods

Strain isolation and screening

The sources of lactic acid bacteria (LAB) were from "tempeh", a fermented soybean cake, "tempoyak", which is a product of fermented durian pulp and "tapai", an alcoholic delicacy made from glutinous rice or cassava.

Lactic acid bacteria strains were isolated from "tempeh", "tempoyak" and "tapai" by weighing 10 grams of each samples and added into 90 ml of bacteriological peptone water (Oxoid). After homogenization, a tenfold serial dilution of the

samples were made and appropriate dilution were streaked on de Man, Rogosa and Sharpe (MRS)-0.14% sorbic acid (MERCK) agar plates. The plates were incubated for 3 days under anaerobic condition at 30°C by placing a gas pack in the anaerobic jar.

Screening of bacteriocin from LAB involves two methods. The first one was to select plates that contain medium density of LAB colonies (30 to 60 colonies) and overlayered them with MRS-soft (0.75%) agar containing indicator strains. The second method was to select the colonies at random, stabbed them onto MRS agar and incubated overnight, anaerobically. The plates were then overlayered with MRS-soft (0.75%) agar seeded with indicator strains. The indicators involved in this study were *Lactobacillus plantarum* 13-2, *Pediococcus acidilactici* 4-46 and *Enterococcus faecalis* N-I-103. These strains were obtained from Microbiology Laboratory, Faculty of Food Science and Biotechnology, UPM. The plates then incubated under anaerobic condition at 30°C. The colonies with antagonistic activity against the indicators were kept in nutrient broth with 15% glycerol and stored in –20°C or streaked on MRS agar for identification and characterization of inhibitory compound.

Identification of lactic acid bacteria

Lactic acid bacteria were characterized by Gram staining and catalase reaction using 30% hydrogen peroxide. The morphology and the motility of the strain were observed under phase contrast microscope. Growth of LAB at 10°C, 15°C and 45°C were done using MRS broth (MERCK). Production of gas from glucose and gluconic acid, arginine hydrolysis, growth in MRS-6.5% broth, growth in acetate agar (5), production of acid and slime from sucrose and final pH in La broth (6) were also determined.

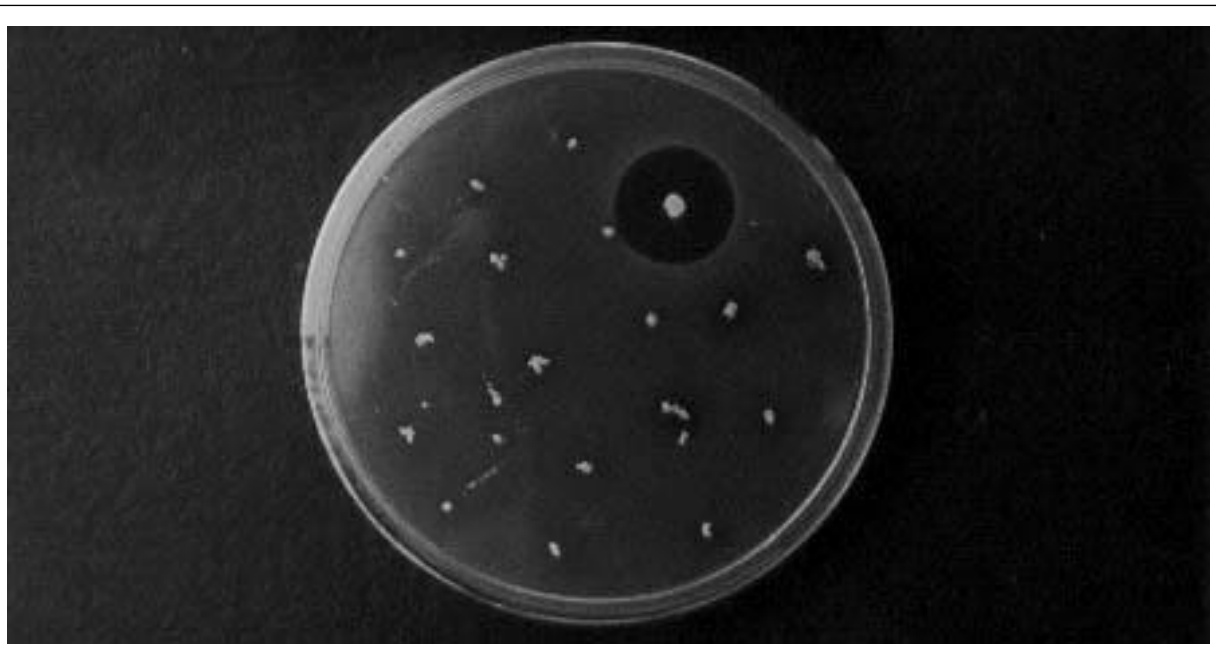
Fermentation of carbohydrates were determined by using API50CHL kit (Biomeriux, France) and the result were analysed by API LAB Plus.

Determining the sources of inhibitory activity

Lactic acid bacteria are able to produce several antimicrobial compounds. These include hydrogen peroxide and organic acids. Bacteriophage may also caused the inhibitory activity. Therefore, it is important to eliminate the inhibition by these compounds in order to ensure that the inhibition was caused by the bacteriocin only. Inhibitory activity by acids can be reduced by using MRS-0.2% glucose (1). Furthermore, preparation of cell free supernatant at pH 6.5 can eliminate the effect of acids produced by lactic acid bacteria against indicator strains.

To eliminate the possibility that hydrogen peroxide may caused the inhibition, a neutralized cell free supernatant were prepared, catalase (5 mg/ml, Sigma) were added into the supernatant before testing for the inhibitory activity. Incubation of

Figure 1: A colony indicated by arrow shows inhibitory zones against indicator strain Enterococcus faecalis *N-I-103*.



producer strain in anaerobic condition may also reduce the effect of hydrogen peroxide against the indicators (7).

The possibility of bacteriophage to cause the inhibitory action was tested by Flip plate method (1). The producer strain was streaked on agar plate and grew anaerobically at 30°C overnight. The agar was then flipped onto the lid of the plates. The indicators were then streaked transversely on the agar and incubated anaerobically at 30°C.

Cell free supernatant

Cell free supernatant were prepared based on methods by Schillinger *et al.* (7). The culture extract of the producer strain were obtained from 18 hours culture grown in MRS broth. The cultures were then centrifuged at 6000 rpm for 10 minutes. The supernatant were adjusted to pH 6.5 using 10N NaOH and filtered through 0.22 µm millipore (Minisart, Sartorius). The resulting supernatant were used for further experiment or stored in –20°C prior to use.

Agar well diffusion assay

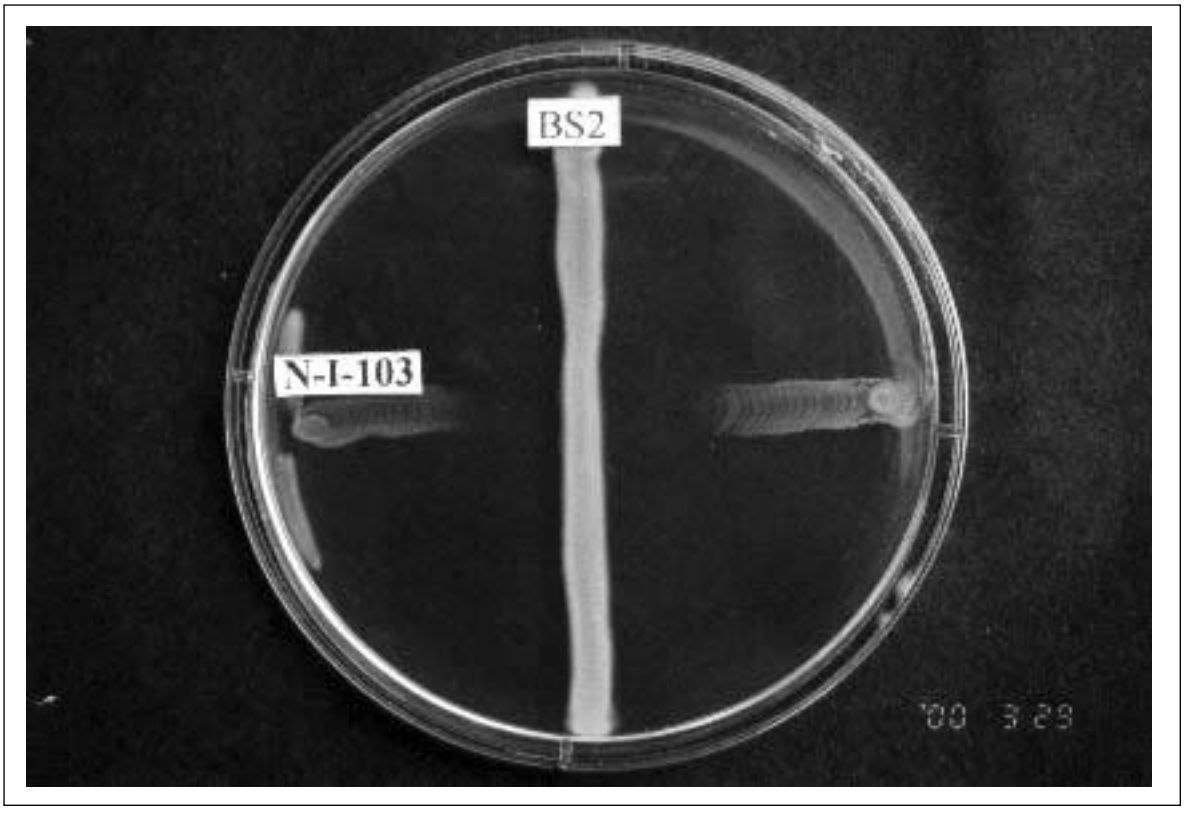
Agar well diffusion methods was suggested by Tagg and Mc Given (8) was used. Wells (5 mm) were made on MRS agar plates using cork borer. 2 to 3 drops of molten MRS agar were added to seal the bottom of the wells and left to hardened. 20 ml of CFS were added into the wells. The plates were kept at –4°C for 2 hours to allow the supernatant to diffuse. The plates were then over layered with MRS-soft (0.75%) agar inoculated with approximately log 7 cfu/ml of indicator (*E. faecalis* N-I-103). The plates were incubated at 37°C overnight.

Activity assay

Serial twofold dilutions of cell free supernatant were prepared using 0.85% sterile saline solution. 20 ml of each of the serial dilution were added into the wells to test for the bacteriocin activity. The activity was assayed by calculating the reciprocal of the highest dilution showing definite inhibition and expressed as Arbitrary Unit /ml (AU/ml) (2).

Time course for bacteriocin production

Figure 2: Flip-plate assay performed to eliminate the involvement of bacteriophage in causing inhibition zone. Therefore, the growth inhibition zone showed by the arrow indicated that the inhibition against Enterococcus faecalis *N-I-103 was due to inhibitory compound produced by* L. plantarum *BS2.*



One percent of overnight culture was inoculated into MRS broth. The culture was then incubated at 30°C. At 2 hours intervals, the growth of the culture were monitored by colony counting at appropriate tenfold dilution to obtain cfu/ml and by optical density at 540 nm. The bacteriocin activity at each time intervals were also determined.

Effect of enzymes towards bacteriocin

The cell free supernatant prepared as above and containing 200 AU/ml of the bacteriocin was used. Supernatant fluid was treated for 1 hour at 37°C with α -amylase, α -chymotrypsin, β-chymotrypsin, lipase, proteinase-K, trypsin, papain and lysozyme (Sigma) at final concentration of 1 mg/ml. The mixture were then heated in boiling water for 3 minutes to denature the enzymes, before testing the bacteriocin activity.

Results and discussion

Isolation and screening

From more than 3000 colonies tested, only one colony was found to produce inhibitory zone. The colony was isolated from "tempeh" and was detected through stab method **(Figure 1)**

Identification of LAB with inhibitory activity

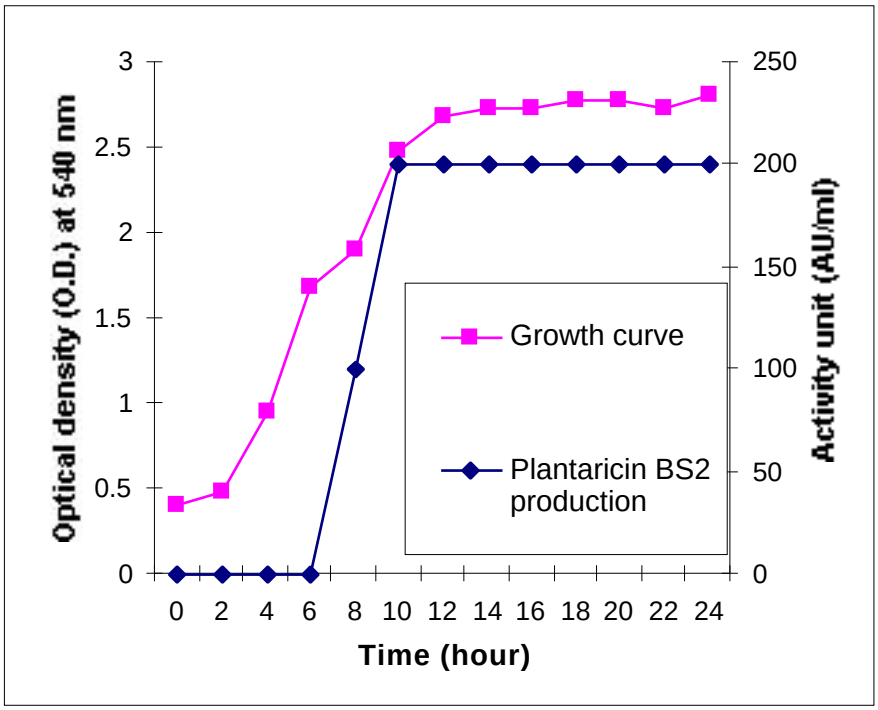
The bacteriocin which exhibited inhibitory activity was found to be Gram positive, catalase negative, short rod and non motile. The culture was able to grow at 10°C, 15°C, 45°C and on acetate agar. It hydrolysed arginine, produced no slime on sucrose agar and gas production from glucose and gluconate. Ability to grow in MRS-6.5% NaCl and in pH 9.6 were other characteristics of this producer strain. These results showed that the isolate was homofermentative *Lactobacillus* species.

The sugar fermentation pattern is presented in **Table 1**. Analysis of the pattern by API LAB Plus shows that it was a *Lactobacillus plantarum* and was given the designation *Lactobacillus plantarum* BS2

Sources of inhibitory activity

Elimination of acid in cell free supernatant does not have any effect on the inhibitory activity of the culture. Addition of catalase reduced the hydrogen peroxide secreted by LAB into H_2O and O_2, therefore reduce the effect of this compound towards the indicators. Positive results after addition of catalase shows that inhibition is not caused by hydrogen peroxide. Clear zone observed from flip plate method **(Figure 2)** shows that bacteriophage is not the source of inhibition since bacteriophage cannot diffuse through agar. Therefore, it can be concluded that the inhibitory action is caused by bacteriocin. This is supported by the effect of proteinase enzymes against the cell free supernatant

Figure 3: Growth curve and bacteriocin production by *L. plantarum* BS2.



(Table 2). Inactivation by all proteinase enzymes tested shows that it is protein or peptide compound. The inhibitory activity of cell free supernatant was also inactivated by addition of α-amylase which showed that this bacteriocin consist of glucidic moieties. The result of enzymes activity against cell free supernatant of *L. plantarum* BS2 are similar to plantaricin UG1 produced by *L. plantarum* UG1 (9)

According to the standard nomenclature for bacteriocin (10) the bacteriocin produced by this bacteria is named as plantaricin BS2. Other reports on plantaricin production by *Lactobacillus plantarum* were by Kato *et al*. (11), Jimenez *et. al*. (12), and Kelly *et al.* (13). However, the comparison of characteristics between plantaricin BS2 and the reported plantaricin are yet to be determined.

Activity assay and time course of bacteriocin production.

The maximum bacteriocin production of 200 AU/ml against *E. faecalis* N-I-103 was achieved after 8 hour of cultivation. Production of plantaricin BS2 started during exponential phase i.e. after 6 hours of incubation. The production reached its maximum when the growth enters the stationary phase **(Figure 3).** This maximum activity was maintained up to 24 hours of incubation.

Application of bacteriocin in food industry

Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* is currently being applied for preservation of dairy products, fish products meat and meat products (14,15,16). Generally, nisin is applied to replace nitrate, a common chemical preservatives used to prevent outgrowth of Clostridia spores as well as other contaminating bacterial pathogens. Currently, bacteriocin had been studied for its suitability to preserve foods. This is due to current concern of the usage of nitrite, which has the potential to form carcinogenic N-nitrosamine. Some biopreservation techniques have now been employed and these involved the introduction of a competitive microflora of lactic acid bacteria (LAB) as protective culturess for chill-stored ready-to-eat meat products, including bacteriocin-producing LAB, and the use of purified anti-listerial bacteriocins added directly as natural food additives. From the results obtained in this study, it is concluded that the *Lactobacillus plantarum* BS2 could serve as a potential source of bacteriocin for use as biopreservative in foods. However, a more detail study on the physical and chemical properties, mode of action and activity against various spoilage and pathogenic bacteria are necessary if the potential of plantaricin BS2 as biopreservative is to be exploited.

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