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Plasmid DNA Analysis of *Pasteurella multocida* Serotype B isolated from Haemorrhagic Septicaemia outbreaks in Malaysia

Jamal, H.*, Chua, K. H., Frederick, D., Mahmood Ameen, A. and Salmah, I.

Molecular Bacteriology Laboratory, Department of Molecular Medicine,
Faculty of Medicine, University of Malaya 50603 Kuala Lumpur, Malaysia.
Email: jamalum2004@yahoo.com

Abstract

A total of 150 purified isolates of *Pasteurella multocida* serotype B were used (Salmah, 2004) for plasmid DNA curing experiment to determine hyaluronidase activity, antibiotic resistance pattern (ARP) and mice lethality test (LD_{50}) for their role of pathogenicity. A plasmid curing experiment was carried out by using the intercalating agent; ethidium bromide and rifampicin, where it was found all the plasmids had been 'cured' (plasmidless) from *Pasteurella multocida*. All of these plasmidless isolates maintained their phenotypic characteristics. They showed the same antibiotic resistance pattern as before curing, produced hyaluronidase and possessed lethality activity in mice when injected intraperitoneally (i.p). Based on this observation, the antibiotic resistance, hyaluronidase activity and mice virulence could probably be chromosomal-mediated. Plasmids were detected 100% in all *P. multocida* isolates with identical profile of 2 plasmids size 3.0 and 5.5 kb. No large plasmids could be detected in all isolates. Since all the isolates appeared to have identical plasmid profiles, they were subjected to restriction enzyme (RE) analysis. From RE analysis results obtained, it can be concluded that the plasmid DNA in serotype B isolates are identical. Only 4 of 32 REs were found to cleave these plasmids with identical restriction fingerprints; *Bgl*II, *Hae*III, *Rsa*I and *Ssp*I. From RE analysis results, it can be concluded that the plasmid DNA isolates are identical. This plasmid might not played any role in pathogenicity of *Pasteurella multocida* serotype B, however this information is important for the construction of shuttle vectors in genetic studies of the pathogenicity of haemorrhagic septicaemia (HS).

Keywords: *Pasteurella multocida* serotype B, plasmid, haemorrhagic septicaemia, hyaluronidase, pathogenicity.

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute septicaemic pasteurellosis, caused by *Pasteurella multocida* (serotype B), which principally affects cattle and water buffaloes. The disease continues to be the major cause of mortality of cattle and buffaloes in Malaysia. Losses in West Malaysia were estimated at about M$ 1.5 million in 1966 (Thomas, 1972) with an average annual lose estimated at M$ 200, 000 for 1967-1976 (Joseph, 1979). The disease occurs in many parts of the world, predominantly in the tropics. Two cases of latest occurrence of HS in Malaysia have been both reported in Terengganu state: Kuala Terengganu (2000) and Rantau Panjang (2001) in which the disease was confirmed to be caused by *Pasteurella multocida* serotype B.

Plasmid has been used in several studies in order to learn more about the pathogenicity and virulence mechanism (Shivshankara, 2000) of the *P. multocida.* Plasmid profile analysis is a useful tool in epidemiological studies (Salmah, 1997, 2000 and 2004). Various studies have been done on *P. multocida* plasmid; in one such studies a cryptic plasmid of *P. multocida* related to its protein has been reported by McGee (2001), elsewhere Plasmid DNA has been used as a probe to identify the species by Zhao and Aoki (1992).

The aim of this study was to characterize the plasmid DNA in *P. multocida* obtained from HS outbreaks by restriction endonuclease digestion. The finding of this preliminary study would help to further investigate whether any of these plasmids are related to virulence mechanism (pathogenicity) of *P. multocida*.

***Corresponding author**

MATERIALS AND METHODS

Bacterial isolates and media

All *P. multocida* isolates used in this study were collected from bovine (cattle, cows and buffaloes) from different outbreaks of HS at different time (year of 1988, 1989, 1990, 1991, 1999, 2000 and 2001) and locations (Malacca, N. Sembilan and Kelantan) in Malaysia. A total of hundred fifty (150) *P. multocida* isolates of pure cultures were collected from Veterinary Diagnostic Laboratory, Petaling Jaya. Isolates were subcultured once onto Brain Heart Infusion (BHI) blood agar, incubated at 37°C overnight before amplified in BHI broth.

Once a *P. multocida* isolate was identified, it was transferred to a fresh bijou bottle BHI agar slant, labelled and stored until used for its phenotypic characterization such as hyaluronidase detection, antibiotic sensitivity testing, mice lethality test (LD_{50}) and plasmid DNA analysis.

Hyaluronidase detection by rapid plate method

A culture-drop and streaking plate methods were used in this study. The plate cultures were prepared as mentioned earlier (Salmah, 1997). These plates were flooded with 2N acetic acid for 10 minutes. The non-degraded substrates precipitate as a conjugate with the albumin, leaving a clear zone around those colonies which produce soluble enzyme that attack the hyaluronate. Hyaluronidase activity is well known among coagulase positive staphylococci; therefore *S. aureus* was used as a positive control in this experiment.

Antibiotic susceptibility testing by E-test

All animal isolates of *P. multocida* were subcultured onto BHI agar plates and antibiotic susceptibility was done using E-test strips obtained from AB Biodisk, Sweden. Briefly, *P. multocida* inoculum was prepared in BHI broth (Difco) and turbidity was adjusted to MacFarland 0.5 turbidity standard. The inoculum was seeded onto Mueller-Hinton blood agar with 7% defibrinated sheep blood. Inoculated plates were incubated at 37^{o}C for 18 hours. The MIC values were read at the end point of intersection between the zone edge and the E-test strip. The cut off values to decide antibiotic susceptibility in E-test were according to the manufacturer prescription.

Mice lethality test (LD_{50})

Mice lethality test was carried out as described by Jamal (2004). The inoculum was prepared from a brain heart infusion broth culture of *P. multocida*, which had been incubated at 37^{o}C for 18 hours. The broth culture was then centrifuged and the cells were resuspended in Phosphate buffered saline (PBS). Mice were injected with 0.5ml of 10^{7} viable organisms intraperitoneally. The virulence (lethality) observation was made for 18-72 hours.

Plasmid curing

The procedure described by Caro *et al*., (1984) with modification was followed, using both ethidium bromide and rifampicin as chemical curing agents. The test organism was grown in BHI broth supplemented with ethidium bromide and rifampicin (final concentration 25-100 µg/ml, respectively). Both sets were incubated at 42°C for 18 hours. Culture tube with highest concentration of the dye and showing visible growth was selected and was ten-fold diluted in fresh BHI broth, 500 µl of the dilutions were spread onto BHI agar plates with the help of a sterile spreader. After overnight incubation at 37°C, colonies were screened for loss of plasmid DNA, antibiotic resistance pattern and mice lethality test (LD_{50}). All the results obtained were compared before and after curing experiment.

Plasmid DNA detection

All the isolates were grown overnight at 37^{o}C in 3 ml BHI broth. Plasmid DNA was extracted by using Promega Wizard© Miniprep Plasmid DNA Purification Kit, in which the procedure was done according to the manufacturer's instruction. The presence of plasmid DNA after curing was detected by agarose gel electrophoresis.

Plasmid DNA profiling and restriction enzyme analysis

Plasmid DNA profiling and agarose gel electrophoresis were carried out as described recently (Salmah, 2004) against 150 strains isolated from outbreaks cases of HS in animals. Thirty (30) restriction endonuclease enzymes were tested for their ability to cleave the plasmids that have extracted from all the 150 *P. multocida* HS-causing isolates. The restriction endonucleases *Ssp*I, *Hae*III, *Rsa*I, *Bgl*I, *Bgl*II, *Hpa*II, *Kpn*I, *Hpa*I, *Eco*RI, *Eag*I, *Cla*I, BclI, *Bam*HI, *Ava*I, *Ava*II, *Apa*I, *Acc*I, *Msc*I, *Nde*I, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sca*I, *Sma*I, *Sph*I, *Xba*I and *Xho*I were used under conditions recommended by the supplier (New England Biolabs. Inc., U.S.A. and Bethesda Research Laboratories, U.S.A.). Restricted DNA (0.2 to 1.0 µg) was subjected to electrophoresis in 1.2% agarose gels.

RESULTS AND DISCUSSION

Phenotypic Characterization

Bacterial Morphology

Gram-stained preparations from all cultures varied from uniform arrangements of Gram-negative short coccobacillary-shaped rods occurring singly, in pairs and occasionally in short chains, to preparation of Gram-negative rods of varying lengths.

The *P. multocida* colonies were 0.5 to 1.0 mm in diameter and smooth with entire edge, increasing in size to 1.0 to 2.0 mm with continued incubation at 37°C on BHI blood agar. Colonies were grayish-yellow, smooth, glistening and transparent with a butyrous consistency (Figure 1).

Hyaluronidase production

Hyaluronidase production was only demonstrated in all of the isolates that had been recovered from cases of HS (Figure 2). Similar findings were also reported in earlier study by Carter and Chengappa (1980), in their survey of 74 cultures of *P. multocida*, representing serotype A, B, D and E had shown that only each of isolates serotype B of *P. multocida* from cases of HS produced hyaluronidase.

The fact that only the type B HS cultures produced hyaluronidase is particularly interesting in that these cultures are known to be highly virulent. However, before one can conclude that only the type B HS cultures produced hyaluronidase, abundance of type B cultures

and also type B from wound cases should be examined closely. If it is ultimately confirmed that only the type B HS isolates produce hyaluronidase, this characteristic may be of value in their identification. Whether or not this enzyme has a role in the pathogenesis of HS seems worthy of investigation.

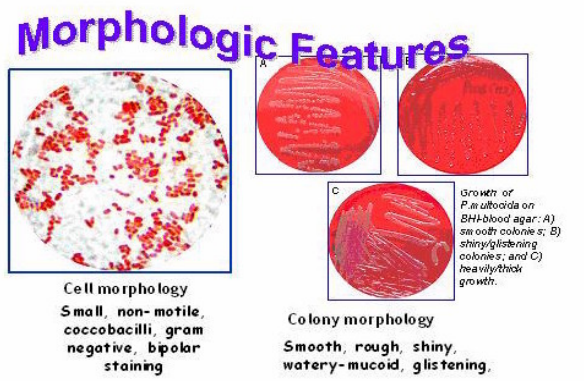

Figure 1: Cell and colony morphology of *Pasteurella multocida* isolated from HS outbreaks.

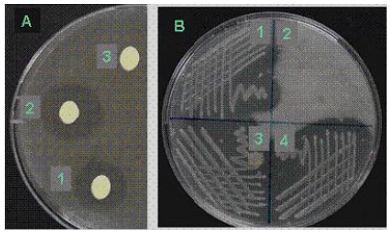

Figure 2: Rapid plate method of hyaluronidase test; (A) culture-spot method of *S.aureus* ATCC25933 (1) as a positive control; representative isolates of *P. multocida* serotype B (2) and *E.coli* ATCC 25922 (3) as a negative control; B) streaking method of *S.aureus* ATCC25933 (1) as a positive control; *E. coli* ATCC 25922 (2) as a negative control; and representative isolates of *P. multocida* serotype B (3, 4).

Antibiotic resistance pattern (ARP)

The antibiotic resistance phenotypes of the isolates in this study indicate that the *P. multocida* are almost always resistant to sulphonamides and spectinomycin irrespective of the serotype, time or location of isolation. All isolates were susceptible to chloramphenicol (Cm), erythromycin (Em), gentamicin (Gm), kanamycin (Km) and trimethoprim + trimethoxazole (Sxt). In this study, it was found that sulphonamides and spectinomycin were ineffective against *P. multocida* isolates. It therefore appears, that the *P. multocida* in Malaysia are generally more sensitive to the antibiotics tested compared to isolates studied elsewhere on the basis of the susceptibility results in this study. The antibiotics that can be considered for use against *P. multocida* are chloramphenicol, erythromycin and the aminoglycosides gentamycin and kanamycin.

Mice lethality test (LD_{50})

It was found that all isolates of *P. multocida* serotype B were virulent to mice and caused death within 18 hours. The histo-pathological examination showed the typical HS symptoms (Figure 3).

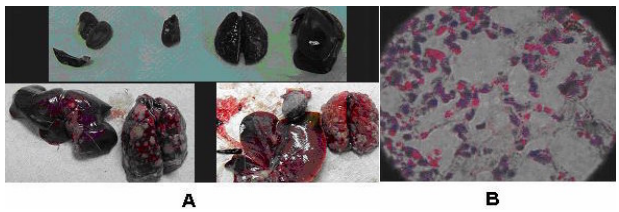

Figure 3: Histopathological analysis of *P. multocida* serotype A- Gross pathology and B-Histo-slide.

Molecular Characterization

Detection of Plasmid DNA

One hundred and fifty isolates of *P. multocida* were examined for the presence of plasmid DNA. All *P. multocida* isolates showed 2 plasmids bands of 3.0 and 5.5 kb in size (Figure 4). Plasmid profile analysis indicated that all isolates of *P. multocida* harboured plasmids with identical reproducible patterns on repetitive testing.

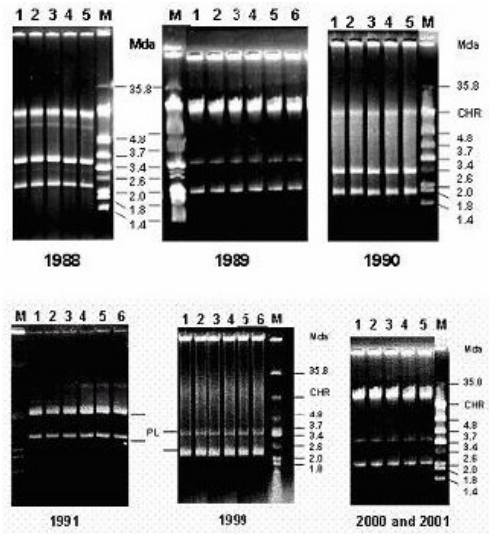

Figure 4: Agarose (1.0 %) gel electrophoresis of plasmid DNA extracted from representative of *P. multocida* isolated from HS outbreak in 1988 - 2001. *E. coli* V517 was used as molecular weight marker (Lane M).

Restriction Endonuclease (RE) Analysis

Irrespective of the geographical distribution and the time of occurrence all the isolated *P. multocida* which cause HS disease in cattle and buffalos had common RE profile .All these plasmids contain a single unique site for *Hae*III, which is four-base cutter enzyme. The recognition sequence for *Hae*III is rich in bases guanine (G) and cytosine C, while *Bgl*II, *Rsa*I and *Ssp*I recognizes a DNA sequence that contains more adenines (A) and thymines (T) than Gs and Cs. These results indicate that *P. multocida* plasmid DNA contains more As and Ts than Gs and Cs. (Figure 5). Studies have indicated that the G-C content of *P. multocida* DNA was 40.3% (Berman and Hirsh, 1978) which means that the DNA is A-T rich. From RE analysis results, (Table 1) it can be concluded that the plasmid DNA isolates are identical. This strongly suggests that isolates of *P. multocida* from all HS outbreaks in Malaysia had only a single predominant plasmid profile and may have originated from a common ancestor.

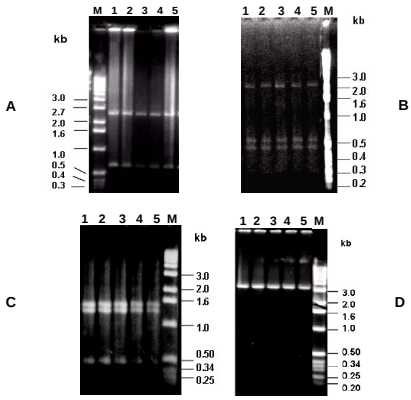

Figure 5: Agarose (1.2%) gel electrophoresis of plasmids DNA of *P. multocida* HS isolates digested with *Bgl*II (A), *Rsa*I (B), *Ssp*I (C) and *Hae*III (D).

Table 1: Restriction endonuclease digestion of *P. multocida* isolates from haemorrhagic septicaemia outbreaks.

RE Analysis	No. of cutting sites	Size of fragments (kb)	Total (kb)
*Bgl*II	2	2.4, 0.6	3.00
*Hae*III	1	3.0	3.00
*Rsa*I	3	2.0, 0.55, 0.45	3.00
*Ssp*I	3	1.30, 1.20, 0.5	3.00

Plasmid Curing

This study was carried out to detect the virulence determinant of *P. multocida* serotype B isolates causing HS whether is plasmid- or chromosomal-mediated. Therefore, *P. multocida* was subjected to plasmid curing via ethidium bromide and rifampicin. Once the curing experiment was successfully done, the isolate was subjected to plasmid screening, antibiotic resistance pattern and mice lethality test. Data (Table 2) shows the results obtained from the curing experiment. It was found all *P. multocida* isolates were able to cure plasmid by ethidium bromide and rifampicin with the least concentration of 50µg/ml. Both of the agents successfully cured the plasmid at concentration of 100µg/ml (Figure 6).

Table 2: Effect of ethidium bromide and rifampicin in plasmid curing experiment of *Pasteurella multocida* serotype B isolates.

Representative isolate	Ethidium bromide (µg/ml)			Rifampicin (µg/ml)		
	25	50	100	25	50	100
Growth condition	+++	++	+	+++	++	+
Colonies per plate	300	50	8	280	38	10
Colonies screened for plasmid	150	50	8	140	38	10
Colonies plasmidless	0	26	8	0	15	10

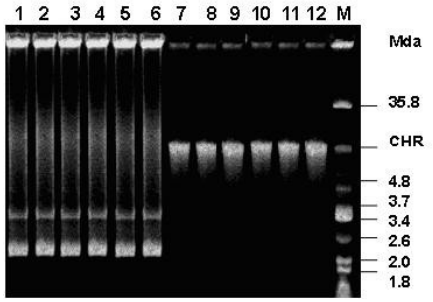

Figure 6: Agarose (0.7%) gel electrophoresis showing plasmid DNA extracted from selected colonies of representative isolates of *Pasteurella multocida* serotype B in curing experiment. Lane 1 – 6: before plasmid curing experiment; Lane 7 – 12: after plasmid curing experiment and Lane M: molecular weight standard marker, *E. coli* V517 used in this study.

CONCLUSION

In this study, it was observed that all the plasmid-cured isolates retained the same antibiotic resistance pattern, hyaluronidase activity and mice lethality test as before the curing experiment (Table 3). This shows that plasmid(s) in *P. multocida* might not carry any virulence gene, hyaluronidase activity and antibiotic resistance, which are most likely on the chromosome. From these findings we concluded that, plasmid does not play any role in the pathogenicity of *Pasteurella multocida* serotype B. Even though these cryptic plasmids do not code for any 'useful' product, they can be involved in the construction of shuttle vectors in genetic studies of the pathogenicity of HS.

Table 3: Effect of curing experiment on phenotypic characteristics of *P. multocida* isolates.

Phenotypic characteristics	Curing Experiment	
	Before	**After**
Antibiotic Resistance Pattern	S_3SH, S_3SHTe, SmS_3SHTe, AmS_3SH, AmS_3SHTe, SmS_3SH, AmSmS3SH, $AmSmS_3SHTe$	S_3SH, S_3SHTe, SmS_3SHTe, AmS_3SH, AmS_3SHTe, SmS_3SH, AmSmS3SH, $AmSmS_3SHTe$
Hyaluronidase activity	All positive	All positive
Mice Lethality Test (LD_{50})	All lethal	All lethal

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