Molecular Characterization of *Pasteurella multocida* Isolates that Cause Haemorrhagic Septicaemia in Thailand using Pulsed-field Gel Electrophoresis

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Abstract

    Pasteurella multocida causes haemorrhagic septicaemia (HS), which is a severe epidemic disease in cattle and buffaloes. In this study, serological typing, minimum inhibitory concentration (MIC) and pulsed-field gel electrophoresis (PFGE) were used to characterize 87 HS isolates caused by *P. multocida* from cattle and buffaloes during 1989 to 2011 in comparison with Thai and Laotian vaccine strains and the reference *P. multocida* strain P1256. The results of serotyping showed that the majority of the isolates (88.5%) belonged to serotype B: 2 (n = 77) while 11.5% shared with serotype B: 2, 5 (n = 10). MIC results showed that 100% of the isolates were sensitive to enrofloxacin and tilmicosin with MIC90 at 0.25 and 8 µg/ml, respectively, while 100%, 67.8% and 20% resistance were found to penicillin G, tetracycline and doxycycline with MIC90 at 64, 8 and 2 µg/ml, respectively. *Apa*I-digested PFGE revealed a high degree of homogeneity. Eighty one isolates were PFGE type 4 (93.10%), as well as the Thai and Laotian vaccine strains, 4 isolates were PFGE type 1 (4.60%), 1 isolate was PFGE type 2, and 1 isolate was PFGE type 3. The reference *P. multocida* strain P1256 belonged to PFGE type 2. In conclusion, a remarkable homogeneity was observed among the HS isolates caused by *P. multocida* in Thailand over the past 22 years.

Introduction

Haemorrhagic septicaemia (HS) is an acute fatal septicemic disease of cattle and buffaloes, caused by *Pasteurella multocida*. A particular group of serotypes associated, B: 2 in Asia and E: 2 in Africa (Rimler and Wilson, 1994). The disease is endemic in most parts of tropical Asia, Africa and India. The disease is still considered as one of the most economically important livestock diseases of South East Asia (Carter and De Alwis, 1989) where cattle and buffaloes are raised mainly as draught animals in rice fields, but to a lesser extent, for milk production (FAO, 1994, 1995). Where animals are used for draught power, they are managed in an extensive free-range system. Such conditions are an ideal environment for the spread of the disease. Such animals are often less well managed, with lower vaccination coverage than more intensively farmed animals. In addition, smuggled cross border animal trade promotes the disease outbreaks because the movement of animals can precipitate new outbreaks of the disease in two ways. Firstly, the animals may be carriers and may infect susceptible stock. Secondly, the animals moved may be susceptible to the disease and become infected from native immune carriers. In either case, explosive outbreaks can result (De Alwis, 1999).

P. multocida has a unique capability to infect a wide range of animal hosts with a broad spectrum of diseases, namely HS, fowl cholera and infectious atrophic rhinitis in pigs. Numerous attempts have been made to correlate specific *P. multocida* serotypes with a particular disease. However, serological typing methods have also been found to provide limited characterization of isolates and are not capable of differentiating strains to the extent required for epidemiological studies (Wilson et al., 1992). The use of novel molecular techniques overcomes these limitations. Ribotyping and field alternation gel electrophoresis (FAGE) have been proved useful for differentiation of bacterial strains that have been denoted as identical by all previous typing methods (Hector et al., 1992; Wei et al., 1992; Townsend et al., 1997[a]). In addition, ribotyping was shown to correlate well with restriction endonuclease analysis (REA) typing and has benefit of allowing easier interpretation by highlighting rRNA gene polymorphisms (Snipes et al., 1989), whereas pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) were used to analyze epidemiology of serial fowl cholera outbreaks (Kardos and Kiss, 2005). The aim of this study was to analyze PFGE profiles of *P. multocida* isolates causing HS outbreaks in Thailand in comparison with vaccine strain in order to analyze the epidemiology and vaccine matching. Additional minimum inhibitory concentration against antimicrobial drugs was evaluated and served as drugs of choice for HS.

Materials and Methods

Bacterial strains: Ninety two strains of *P. multocida* were employed in this study. They were 87 Thai field isolates of HS from cattle (n = 16) and buffaloes (n = 71) collected from 1989 to 2011 in the culture collection of Bacteriology and Mycology Laboratory, National Institute of Animal Health (NIAH), Thailand. In addition, field isolates of *P. multocida* serotype A: 1 from cattle (n = 2), a Thai vaccine strain used for production of HS vaccine by Department of Livestock Development (n = 1), a Laotian vaccine strain (n = 1), and a reference *P. multocida* strain P1256 (B: 2) associated with HS in buffalo in Asia from the National Animal Disease Center were adopted for comparison.

Bacterial isolation and identification: The Thai *P. multocida* field isolates were isolated from cattle and buffalo samples collected at necropsy in NIAH and our 7 regional Veterinary Research and Diagnostic Laboratories. The samples were identified by cultural characteristics and biochemical tests (Quinn et al., 1994). All isolates were routinely streaked for purity on blood agar (Oxoid, England), incubated overnight at 37°C with 5% CO_2, and stored in Mist desiccans (Fry, 1951) at -70 to -80°C and parallel with storing as lyophilized ampoules at 4°C until used.

P. multocida serotyping: Capsular typing (A, B and D) was performed by employing indirect hemagglutination (IHA) test (Sawada et al., 1982) and somatic serotyping (1-16) was performed by gel diffusion precipitin test, GDPT (Brogden et al., 1977). Antisera against capsular type A, B and D were prepared from New Zealand white rabbits, while antisera against somatic type 1-16 were prepared from white leghorn chicken as previously described by Tanticharoenyos et al. (1993)

Antimicrobial susceptibility of P. multocida: Minimum inhibitory concentration (MIC) was determined against 5 antimicrobial agents, doxycycline, enrofloxacin, pennicilin G, tetracycline and tilmicosin (Sigma, USA). The MIC was determined according to Citron et al. (2005). In preparation for testing, the isolates were grown overnight on blood agar at 37°C. A bacterial suspension equal to a McFarland standard of 0.5 was prepared in 0.85% saline and further diluted in cation-adjusted Mueller-Hinton broth (Oxoid, USA) with 5% lysed horse blood (LHB) to a concentration of approximately 10^6 CFU/ml. Fifty microliters of this suspension were inoculated into the wells of the panels, which also contained 50 μl of the antimicrobials at double strength, giving a final inoculum of approximately 5×10^5 CFU/ml or 5×10^4 CFU/well and a final concentration of 2.5% LHB. Incubation was carried out at 37°C in ambient air for 18 to 20 h. After incubation, the growth in the panels was read and the MICs recorded. The MIC was determined to be the lowest concentration that completely inhibited growth of the organism. Reference strains, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213, were used as quality control limits of MICs and interpretation following instructions of CLSI 2007 and 2008.

Pulsed-field gel electrophoresis (PFGE): PFGE was carried out according to Lainson et al. (2002). Briefly,

P. multocida isolates were cultivated in brain heart infusion broth (Oxoid, USA). Then, bacterial cells were pelleted by centrifugation. The bacterial cells were resuspended with Tris-EDTA buffer (10 mM Tris HCl, 10 mM EDTA, 1 M NaCl, pH 8.0) supplemented with 1 mg/ml of lysozyme. Bacterial suspension which adjusted to McFarland no 4 turbidity (4×10^8 cells) was mixed with an equal volume of 2% low melting-point agarose (Bio-Rad, USA). The gel plugs were incubated at 37°C for 4 h in a buffer containing 6 mM Tris HCl, 100 mM EDTA, 1 M NaCl, 0.5% (w/v) Brij 58, 0.2% sodium deoxycholate and 0.5% lauroyl sarcosine supplemented with 1 mg/ml of lysozyme. Subsequently, the plugs were incubated at 55°C overnight in a solution containing 0.25 M EDTA, 20 mM NaCl, 1% lauroyl sarcosine and 1 mg/ml of proteinase K. Then they were washed 3 times in Tris EDTA buffer at 4°C for 1 h. The genomic material (total DNA) was digested with 30 U *ApaI* (New England BioLabs, USA), the digestion conditions were indicated by the producer. The resulted DNA fragments were separated by a CHEF-DRII system (Bio-Rad, USA) in 1% agarose (Bio-Rad, USA). The electrophoresis conditions were as follows: Tris-Borat-EDTA 0.5 x buffer, at 14°C, 6 V/cm of electric field, an angle of 120°

and a linear ramp. For the separation of the macrorestriction *ApaI* fragments migration program lasted 22 h, with a pulse spacing of 1-25 sec. Gels were stainined with 0.5 μg/ml solution of ethidium bromide. Then, relationship between *P. multocida* isolates was analyzed by BioProfile (Vilber Lourmat, Germany)

Results

Results of *P. multocida* serotyping from 87 HS isolates in Thailand, 77 isolates (88.5%) belonged to B: 2, 10 isolates (11.5%) were B: 2.5. The MIC of 5 antimicrobial agents for 87 HS isolates is presented in Table 1. All of the isolates were 100% sensitive to enrofloxacin and tilmicosin with MIC90 at 0.25 and 8 μg/ml, respectively, whereas all of them were resistant to penicillin and 80% and 7.8% were resistant to doxycycline and tetracycline, respectively (Table 1).

The PFGE method with the digestion with restriction enzyme *Apa*I has a power to discriminate the 87 isolates into 4 PFGE patterns. The majority of

Table 1 Minimal inhibition concentration (MIC) of antimicrobial agents (μg/ml) for *P. multocida* causing HS in cattle and buffaloes in Thailand during 1989 to 2011 (n = 87)

Antibiotics	Breakpoints*	%R	%I	%S	%R 95%C.I.	MIC50	MIC90	Geom. Mean	MIC Range
Penicillin G	S≤0.5 R≥1*	100	0	0	94.9-100	64	64	58.35	4 – 64
Doxycycline	S≤0.5 R≥1*	80	0	20	70.0-87.4	1	2	0.992	0.25 - 2
Tetracycline	S≤2 R≥8**	7.8	24.4	67.8	3.5-15.9	2	4	2.245	1 – 16
Enrofloxacin	S≤0.25 R≥2**	0	0	100	0.0-5.1	0.125	0.25	0.136	0.125 - 0.25
Tilmicosin	S ≤16 R≥32**	0	0	100	0.0-5.1	8	8	6.016	1 - 16

* CLSI (formally NCCLS) 2007, ** CLSI (formally NCCLS) 2008
R: resistant, I: intermediate, S: sensitive, Geom. Mean: Geometric Mean

Table 2 PFGE Patterns of 87 HS isolates caused by *P. multocida* in Thailand during 1989 to 2011

PFGE Pattern	No. of isolates (%)	Serotype	Remark and province
PFGE 1	4 (4.60%)	B:2	Internal organs of buffalo from Nakhon sri-thammarat (n=2, strain 26679/32 and 28679/32) Internal organs of buffalo from Phitsanulok (n=1, strain 830/43) Internal organs of cattle from Chiang rai (n=1, strain 1346/43)
PFGE 2	1 (1.15%)	B:2	Internal organs of buffalo from Sakon nakhon (strain 1744/44)
PFGE 3	1 (1.15%)	B:2	Internal organs of buffalo from Phangnga (strain 102361/49)
PFGE 4	81 (93.10%)	B:2 or B:2,5	Cattle (n=66) and buffaloes (n=15) from 16 provinces

them were clustered in PFGE pattern 4 (93.10%) as shown in Table 2. The Thai and Laotian vaccine strains were also in the same PFGE pattern 4, while the reference strain P-1256 was PFGE pattern 2 as well as one isolate from Sakon nakhon (Fig 1). The dendrogram in Fig 2 showed that all of the HS isolates had 84% to 100% similarity and the two *P. multocida* isolates of serotype A: 1 (lane 2, 3) exhibiting 94% similarity. On the contrary, they showed only 62% similarity to the HS isolates.

Discussion

Even though *P. multocida* serotype B: 2 was reported as the causal agent of HS in Asia (Rimler and Wilson, 1994), and serotype E: 2 was reported in Africa (Francis et al. 1980), Egypt and Sudan have recorded the presence of both B and E serotypes (Shigidi and Mustafa, 1979). In addition, serotype B: 3, 4 was isolated from Bison from Montana in 1965, a dairy calf strain in 1969 and a deer calf strain from Canada (Rimler and Wilson, 1994), and deer with septicemia in the United Kingdom in 1982 (Jones and Hussaini, 1982; Myint et al., 1987; Rimler et al., 1987). Thus, serotyping is routinely performed in order to monitor in the epidemiology and to make certain matching of HS outbreak with vaccine strain. In this study only group B, either B: 2 or B: 2, 5, was found to be the cause of HS agent in Thailand, B: 2 being was the majority of them (88.5%). Serological classification of strains into Heddleston serotype 2 and 5 is dependent on the presence or absence of phosphoethanolamine (PEtn) moiety at the 3 position of the second heptose (Hep II) of the capsular lipopolysaccharide structure (Michael et al., 2009).
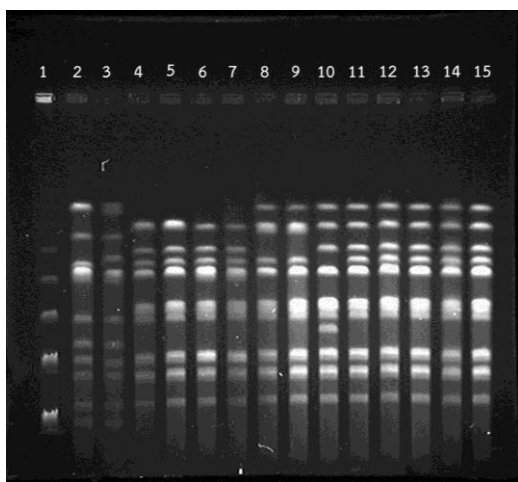
Figure 1 PFGE patterns of HS caused by *P. multocida* in Thailand during 1989-2011 and digested with *Apa*I. Lane 1 Lambda Ladder PFG Marker (BioLabs, USA) used as molecular size markers; Lane 2 and 3, two isolates of *P. multocida* serotype A: 1 from cattle (control), Lane 4-7 pattern 1 from Nakhon sri thammarat (n = 2), Phitsanulok (n = 1) and Chiang rai (n = 1); Lane 8-9, pattern 2, one field isolate from Sakon nakhon and reference strain P-1256; Lane 10, pattern 3, one isolate from Phangnga; Lane 11-13, pattern 4, representing most of the Thai field isolates; Lane 14-15, pattern 4, Thai and Laotian vaccine strains

However, in our study both B: 2 and B: 2, 5 were not different in term of PFGE analysis, while difference in pathogenesis needs further study. Penicillin was recommended as drug of choice for HS treatment and was reported as sensitive for 10 strains obtained from Malaysia, Indonesia, Thailand, Myanmar, India and Sri Lanka (De Alwis, 1984), as well as 16 isolates obtained from different outbreaks in Gujarat State of India (Bandopadhyay et al., 1991). However, it is likely to vary from one country to another depending on prevailing drug usage practices. In this study, the HS isolates showed high resistance to penicillin G, with MIC90 at 64 µg/ml, which is far from the breakpoint for the drug resistance (≥1 µg/ml). In case of tetracycline the MIC90 was 4 µg/ml, which was still below the resistant break point (8 µg/ml). However the proportion of sensitive strains was only 68.7% while the figure of intermediate stains was high (24.4%). Thus, the percent resistance might increase when the drug is used for a prolong period, since the intermediate strains can shift to resistance. In addition, the evidence of tetracycline resistance was emphasized by doxycycline sensitivity, as only 20% of the isolates showed sensitivity to the drug. Thus, drug sensitivity information is useful for deciding the drug of choice for controlling early outbreak in order to minimize losses from HS. According to this study tilmicosin or enrofloxacin are recommended for the treatment of HS under veterinarian prescription.

The Thai HS isolates were mainly in PFGE 4 (93.10%). The finding is consistent with the result of ribotyping as reported by Thownsend et al. (1997[b]), in which the Asian field isolates exhibited identical Eco RI banding patterns, Ribotype I. Following SmaI digestion in field alteration gel eletrophoresis (FAGE) also showed similar finding that most of the HS-isolates originating from Asia were shown to have identical banding patterns. Our recent study using multilocus sequence typing (MLST) and PFGE for comparing the 23 field isolates and vaccine strains of HS isolates from Pakistan and Thailand also supported the fact. It was found that 20 out of 23 isolates tested were MLST Sequence Type 122. The Sequence Type 122 is dominant associated profile with HS cases in South Asia. PFGE results, however, showed one band difference between the Pakistani and the Thai isolates (Moustafa et al., 2013). Furthermore, from this study PFGE 1 consisted of 4 isolates. Two of them were strains 26679/32 and 28679/32 cultured from internal organs of buffaloes in the same outbreak in 1979 from Nakhon sri thammarat, a province in the southern part of Thailand, while the other 2 isolates, strains 830/43 and 1346/43, were isolated in 2000 from internal organs of buffaloes from Phitsanulok and internal organs of cattle from Chiang rai, provinces in the central and northern part of Thailand, respectively. The correlation between the outbreaks was unclear. The power of PFGE together with MIC could be used to discriminate the strains 26679/32 and 286279/32 from the other 2 isolates. As the MIC pattern of the formers for doxycycline, enrofloxacin, penicillin G,
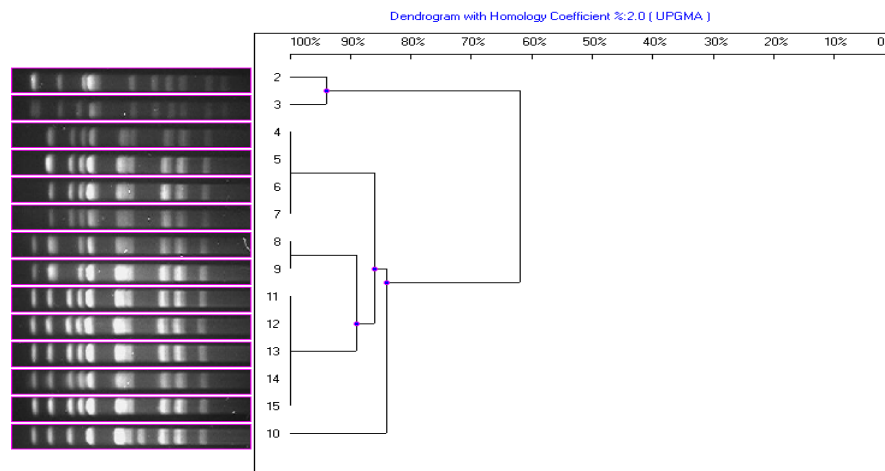
Figure 2 Composite dendrogram showing relationship between *P. multocida* isolates

tetracycline and tilmicosin was 1, < 0.125, 64, 4, 2 µg/ml while MIC patterns of the strains Bu 830/43 and Ca 1346/43 were 1, < 0.125, 64, 16, 2 and 0.5, < 0.125, > 64, 2, 1. Thus, we can conclude that the strains 26679/32 and 286279/32 came from the same origin while the strains Bu 830/43 and Ca 1346/43 were not likely came from the same source. Additionally, the power of PFGE and serotyping can discriminate the strains 26679/32 and 28679/32 from the strain 05779/32. The later was isolated from hearts, livers, spleens and kidneys of dead cattle and diagnosed as HS by Mahitanan et al. (1981). Consequently, we determined that both of the strains 26679/32 and 286279/32 were serotype B:2 and belonged to PFGE 4 while strain 05779/32 was serotype A:1 and had different PFGE pattern. Moreover, the cattle (05779/32) did not die from HS and the disease in the cattle was not derived from buffaloes. Thus, the *Apa*I digested PFGE in this study was useful to discriminate *P. multocida* y serotype A:1 from B:2 HS isolates. The result of PFGE analysis revealed that the HS isolates in Thailand were homogeneous. Interestingly, there was only one HS isolate from internal organs of a buffalo from Sakon nakhon that belonged to PFGE 2, similar to the reference strain P1256 from the National Disease Center collection. The strain was previously reported as serogroup B and the origin was from Asia (Rimler, 1978). In similar manner there was only one isolate that belonged to PFGE 3. The strain was isolated from internal organs of a buffalo from Phangnga. The epidemiology of the PFGE 2 and PFGE 3 is still unclear. Thus, from this study we can conclude that PFGE, serotyping and MIC test can be helpful to discriminate the strains to a certain degree and there is remarkable homogeneity was observed among the HS isolates of *P. multocida* in Thailand over the past 22 years.

Acknowledgements

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บทคัดย่อ

ลักษณะทางชีวโมเลกุลของเชื้อพาสเจอเรลลา มัลโตซิดา ที่เป็นสาเหตุของโรคเฮโมราจิก

เซพติซีเมียในประเทศไทยโดยใช้เทคนิค pulsed-field gel electrophoresis

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พาสเจอเรลลา มัลโตซิดา เป็นเชื้อแบคทีเรียที่เป็นสาเหตุของโรคเฮโมราจิกเซพติซีเมียซึ่งเป็นโรคระบาดอย่างรุนแรงในโคและกระบือ การศึกษาครั้งนี้วัตถุประสงค์ที่จะทำการแยกคุณลักษณะของเชื้อดังกล่าวจำนวน 87 สายพันธุ์ที่แยกได้จากโคและกระบือ ในช่วงปี 2532 ถึง 2554 โดยใช้วิธีตรวจหาซีโรไทป์ ความไวต่อยาต้านจุลชีพ (minimum inhibitory concentration, MIC) และ เทคนิค pulsed-field gel electrophoresis (PFGE) เปรียบเทียบกับเชื้อที่ใช้ผลิตวัคซีนของประเทศไทยและสาธารณรัฐประชาชนลาว รวมทั้งเชื้ออ้างอิงมาตรฐานสเตรน P1256 จากการตรวจหาซีโรไทป์พบว่าเชื้อส่วนใหญ่ (ร้อยละ 88.5) เป็นซีโรไทป์ B: 2 (n = 77) ส่วนที่เหลืออีกร้อยละ 11.5 เป็นซีโรไทป์ B: 2, 5 (n = 10) ผลการตรวจ MIC แสดงให้เห็นว่าเชื้อทั้งหมดไวต่อยาเอ็นโรฟล็อคซาซิน และทิลมิโคซิน โดยมีค่าความเข้มข้นของยาที่สามารถยับยั้งเชื้อได้ร้อยละ 90 ของเชื้อที่ทดสอบ (MIC90) อยู่ที่ 0.25 และ 8 ไมโครกรัมต่อมิลลิลิตร (µg/ml) ตามลำดับ ในขณะที่เชื้อทุกสายพันธุ์ดื้อต่อยาเพนิซิลิน โดยมีค่า MIC90 อยู่ที่ 64 µg/ml สำหรับยาเตตราซัยคลิน และด็อกซีซัยคลินมีค่า MIC90 อยู่ที่ 8 และ 2 µg/ml ซึ่งพบว่าร้อยละ 67.8 และ 20 ของเชื้อที่ทดสอบมีความไวยาดังกล่าวตามลำดับ จากการตรวจ PFGE และตัดด้วยเอ็นไซม์ *Apa*I พบว่าเชื้อส่วนใหญ่ซึ่งมีจำนวนทั้งสิ้น 81 สายพันธุ์ (ร้อยละ 93.10) มีรูปแบบ PFGE เป็น PFGE type 4 เช่นเดียวกับวัคซีน สเตรนของไทยและลาว PFGE type 1 พบทั้งสิ้น 4 สายพันธุ์ (ร้อยละ 4.60) และ PFGE type 2 และ PFGE type 3 อย่างละ 1 สายพันธุ์ ส่วนสเตรนมาตราฐาน P1256 มีรูปแบบเป็น PFGE type 2 กล่าวโดยสรุปคือ เชื้อพาสเจอเรลลา มัลโตซิดา ซึ่งเป็นสาเหตุของโรคเฮโมราจิกเซพติซีเมียในประเทศไทยในช่วง 22 ปีที่ผ่านมามีความเหมือนกันเป็นส่วนใหญ่

คำสำคัญ: เฮโมราจิกเซพติซีเมีย ความไวต่อยาต้านจุลชีพ พาสเจอเรลลา มัลโตซิดา pulsed-field gel electrophoresis ซีโรไทป์

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