TREATMENT OF GILL FLUKE DISEASE IN HYBRID GROUPER

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ABSTRACT

Hybrid grouper is a popular marine culture fish in Sabah, Malaysia. However, the production is limited. One of the factors was due to the gill fluke occur in fish farms. Monogenean parasite was suspected as the main cause of high mortality of hybrid grouper in the fish farm. The present study aimed to determine causative agent of gill fluke infection in hybrid grouper at the fish farm and the effective treatment with formalin. Firstly, the gills of the unhealthy fish was cut out and observed under the microscope. The parasite was identified from characteristics of body shape and internal organs using the digital photographic microscope. 7 out of 28 fish were infected with *Pseudorhabdosynochus* sp. while other does not found any parasite attach on the gill. For the effect of treatment (*in vitro*) experiment, gill filaments with monogenean were transferred and immersed in various concentrations of formalin in the petri dish. High concentration more than 300ppm formalin was effective to gill fluke within 30 minutes. 1000ppm formalin was the most effective because all the parasites were immobilized within 10 minutes. For the acute toxicity of formalin, a total 35 healthy hybrid grouper were used in this experiment and put into 7 containers with different concentrations of formalin. The fish were exposed into each formalin for 1 hour. Formalin up to 500ppm was not toxic to the fish. Finally, it is hoped that the finding of this study can be used to control gill fluke disease in grouper farms.

RAWATAN JANGKITAN *GILL FLUKE* KE ATAS IKAN KERAPU HIBRID

ABSTRAK

Ikan kerapu hibrid adalah sejenis ikan laut yang popular di Sabah, Malaysia. Tetapi pengeluaran adalah terhad kerana jangkitan *gill fluke* ke atas ikan kerapu hibrid. Parasit monogenean disyaki sebagai punca utama penyebab kematian yang tinggi di kalangan ikan kerapu hibrid di ladang ikan. Kajian ini bertujuan untuk menentukan ejen penyebab jangkitan *gill fluke* di kalangan ikan kerapu hibrid dan ujian rawatan menggunakan formalin. Pertama, insang pada ikan yang lemah telah dipotong dan diteliti di bawah mikroskop. Di bawah mikroskop fotografi digital, parasit telah dikenalpasti melalui bentuk badan dan organ dalamannya. Terdapat 7 ekor daripada 28 ikan kerapu hibrid dijangkiti oleh *Pseudorhabdosynochus* sp. sahaja. Ujian *in vitro* kesan formalin terhadap monogenean mendapati bahawa parasit tersebut dapat dicegah oleh 300ppm formalin selama 30 minit. Manakala kepekatan formalin 1000ppm adalah rawatan yang paling berkesan kerana parasit mati dalam masa 10 minit. Bagi ujian ketoksikan formalin, sejumlah 35 kerapu hibrid yang sihat telah didedahkan dengan kepekatan yang berbagai-bagai selama 1 jam. Kepekatan formalin 500ppm dan ke bawah tidak menunjuk ketoksikan kepada ikan. Kesimpulannya, kajian ini berharap agar keputusan dapat digunakan untuk mengawal penyakit *gill fluke* di ladang ternakan ikan kerapu hibrid.

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ppm	part-per million
kg	kilogram
%	percent
km^2	square kilometer
mm	millimeter
°C	degree Celsius
cm	centimeter
ml	milliliters
L	liters
AFA	alcohol-formalin-acetic acid
mins	minutes

CHAPTER 1

INTRODUCTION

1.1 Marine Fish Production in Sabah, Malaysia

The Asia-Pacific region was the world largest producer of fish in both aquaculture and fisheries capture (Simon *et al.*, 2012). According to the U.N. Food and Agriculture Organization (FAO), Asia-Pacific region produces 50% of the world's catch of both marine and freshwater fish, 90% of global aquaculture and exhibit the highest in per capita fish consumption (Food and Agriculture Organization, 2014; Santos, 2015).

The total marine fish production in Malaysia was 14.5 million tones with 30-35kg per capita fish consumption in years 2010 (Simon *et al.*, 2012). In the year 2013, Malaysian consumed more than 50kg of fish per person per year had shown dramatically increase compare to 2010 (Department of Fisheries, 2013). At the same time, the food fish supply in Malaysia has shown an increase of 0.23% compared to 2012 (Department of Fisheries, 2013). The demand for marine fish continues to grow due to population growth, rising incomes in the developing world and expanding urbanization (Msangi & Batka, 2014). However, there was significantly reduced in fish stock from wild in the early 1970's due to the uncontrolled usage, grow of trawl fishery, unsustainable production and environmental changes (Simon *et al.*, 2012; Khatib, 2015). Hence, the development of aquaculture in Malaysia has given a positive impact to wild species at the same time provide a food supply for human consumption.

The development of aquaculture in Malaysia shown a significant increased since 1920's (Food and Agriculture Organization, 2009). In 2014, almost half of all food fish for human consumption was produced through aquaculture. In the same year, Sabah was the highest aquaculture production which is 54% of total production in Malaysia (Department of Fisheries, 2013). Therefore, Sabah was set to become an aquaculture leader in Malaysia.

Geographically, Sabah is surrounded by three rich tropical seas including Sulu, Sulawesi and South China with estimated about 4,315km^2 coastline and longest in Malaysia. Other than that, strong government support, established technology and yet-untapped fisheries resources had make it become the great potential for development in fishing and aquaculture (Rayner, 2013). There are 303 fish farm culture of marine fish in cages found mainly in Tuaran, Sandakan, Kudat, Kuala Penyu and Menumbok (Galid, 2013; Rayner, 2013).

1.2 Grouper and Hybrid Grouper

Grouper is a popular marine culture fish that belongs to the genus *Epinephelus* and family Serranidae. In 2000 and 2010, groupers were included in the top twenty cultures species in Southeast Asia and the Pacific region due to its high demand among consumers. Groupers have been cultured more than 20 years which mainly using floating net cages due to low operational costs. Besides that, groupers mariculture was famous due to their robustness under heavy stocking conditions and rapid growth at high temperature (Pierre *et al.*, 2007). The major production of groupers is conducted in Asian countries including China, Indonesia, Malaysia, Thailand and Philippines (Simon *et al.*, 2012). In Malaysia, Sabah was the main production of groupers.

Groupers are widely distributed in the tropical and subtropical regions and inhabit on coral reefs, estuaries, seagrass beds, rocky, muddy and even sandy bottom (Nagasawa & Cruz-Lacierda, 2004; Pierre *et al.*, 2007). However, fish farms often suffer by the limited availability of wild seed stock supply of groupers due to overfishing and damage to groupers' habitat (Yip, 2012). To solve this problem, Malaysia had been importing fish fry of groupers from others countries but the survival rate was too low and susceptible to disease. To reduce the catch of the grouper fry from wild, in November and December is prohibited by the law of Malaysia to reduce heavy pressure on grouper (Robert *et. al.*, 2002).

Due to high price and scarcity of fingerlings from the wild, aquacultures has initiated aquaculture field in the cultivation of interspecific grouper hybrids and reduce the demand-supply gap of grouper. Hybrids were produced under a process called hybridization, which means crossing of two different species (Rahman *et al.*, 2013). Hybrid groupers have been reported to exhibit higher growth rates which can reach marketable size within seven months (James *et al.*, 1999). Due to genetic improvement, hybrid grouper has characteristic superior to both parents. Hybridization also increases disease resistance, improve environmental tolerance and improve flesh quality (Bartley *et al.*, 2000)

In 2006, the first hybrid grouper was produced in University Malaysia Sabah, Sabah called TGGG which is crossbreed between female Tiger grouper (*Epinephilus fuscoguttatus*) and male Giant grouper *(Epinephilus lanceolatus*) while in 2011 Chanfu was produced which is crossbreed between female Camouflage grouper (*Epinephilus polyphekadion*) and male Tiger grouper (*Epinephilus fuscoguttatus*) were produced in 2011 (Addin & Senoo, 2011). Camouflage grouper and Tiger grouper had often been confused due to the similar color pattern of irregular dark blotches. However, both groupers bring different beneficial effects in aquaculture. Camouflage grouper is disease resistant and environmental stress while Tiger grouper can grow faster under hatchery condition (James *et al.*, 1999). Throughout the growth rate, it was proven that Chanfu can reach marketable size within seven months (James *et al.*, 1999). Hence, Chanfu can bring more economic value to aquaculture.

1.3 Threat of Disease in Grouper

In recent years, mortality of hybrid grouper occurs in Sabah due to disease outbreaks. The disease is defined as a disturbance in normal physiological function or structure of the body or any organ or part of the fish (APEC/SEAFDEC, 2001). Due to intensive in the open culture system, floating net cage can act as a reservoir for parasite and cause high loss of grouper production. The disease or pathogen can easily spread to the next batch of fish stock and within the net cages. Hence, high mortality caused serious socio-economic.

There are main two types of disease which are non-infection disease and infection disease. The causative agents for the non-infection disease are related to environment and management factors (Bondad-Reantaso *et al*, 2001). Environment factors are including oxygen depletion, high organic load, unsuitable temperature and pH change to extreme value. To reduce disease outbreak, fish farms should avoid improper feeding and overcrowding, regularly checking nets and appropriate monitoring the water quality. Besides that, handling of the fish may also cause stress and lead to disease occurrence.

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The heavy mortality due to infectious diseases in grouper includes viruses, bacteria, fungi and parasites (Chuah, 2001). Viral diseases are including Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER) and Iridoviruses (Razak *et al.*, 2014). VNN is the most devastating viral infection in grouper because the mortality reaches up to 100% in 2.5-7.5 cm fish (Nagasawa & Cruz-Lacierda, 2004). Bacterial diseases include *Vibrio*, *Pseudomonas*, *Streptococcus* and *Tenacibaculum* infections. Furthermore, *Ichthyophonosis* is known as fungal infection. Serious problems in parasitic disease were caused by protozoans, didymozoid digeneans, nematodes, caligid copepods, isopods, leeches and monogeneans (Cruz-Lacierda & Erazo-Pagador, 2004). The parasitic disease often occurs when fish fry was transferred to the new environment. Parasitic disease found in gills and body surface sometimes kills fish in a farm.

Sometimes it may be hard to control the disease outbreak, however, prevention is better than cure. To increase and improve fish resources in a fish farm, maintaining the fish farm environment was the most effective way and followed by correctly diagnose and treat the diseased fish using chemotherapeutics in the early stages before the disease spread. Chemotherapeutants refer to any medication, drug or chemical which act as a control or prevention in disease problems and improve the productivity in disease management. Hence, it is important to establish effective control against infection and method for chemical use (Inui, 2002).

1.4 Significance of Study

Recently fish farms are facing disease outbreak at Kuala Peuyu in, Sabah, Malaysia. The hybrid grouper was suspected facing gill fluke disease in the floating net cages. Therefore, it is important to diagnose the fish and find out the causative agent which causes the mortality in the fish farm. If gill fluke disease at fish farms could be identified, an effective treatment method also may be found. The development of an effective treatment method may be improved the aquaculture management at the fish farms. This may be helpful to solve the problem if the same disease occurred in some fish farms.

1.5 Objectives

The main objective of the current study is to provide effective treatment regarding gills fluke disease in grouper.

The specific research objectives include:

1. To determine causative agent of gill fluke disease in grouper at fish farm in Kuala Penyu in Sabah, Malaysia.

2. To establish the method of effective treatment from the relationships between formalin concentration and dipping time to kill the parasite *in vitro.*

3. To determine the toxicity of formalin to hybrid grouper within an effective dipping time.

1.6 Hypotheses

The hypotheses of this study are:

1. Monogenean is a dominant parasite of grouper at the fish farm.

2. Treatment method exposing disease fish to 300ppm formalin for 30 minutes may be effective to kill the parasite.

3. Treatment method exposing to 300ppm formalin for 1 hour may be not toxic to hybrid grouper.

CHAPTER 2

LITERATURE REVIEWS

2.1 Gill Fluke Disease

Flukes are a common term for small flatworm belonging to the Phylum Platyhelminthes (Shaharom, 2002). Most of the species are found on the skin or gills of the fish but rarely found in blood vascular system, eye, ureter and body cavity (Robert *et al.*, 2009). For gill fluke incident, it has been reported in grouper in Malaysia (Nagasawa & Cruz-Lacierda, 2004; Jithendran *et al.*, 2005; Mudo *et al.*, 2014). The gill flukes are ectoparasites with 0.5-1 mm long in size (Katoch & Godara, 2014). Gill flukes can infect in both freshwater and seawater fishes, and both wild and farmed fishes. Fish infected with gill fluke rarely cause disease or death in wild fish. However, gill fluke disease often occurs in farmed fish due to high stocking density and poor filtration system. It is easier for the larvae to find their host to attach within a short time.

Gill fluke infection is hard to visually see on the fish and no obvious symptoms were observed when the fish infected. Hence, fish that infected by gill fluke are often neglected by the fish farms. When the number of the parasite was low, it causes little or no harm to the fish. The gill flukes can cause significant problems and threaten to grouper aquaculture when excessive parasite loads on the gill filaments.

Gill fluke is interrelated with other pathogens such as bacterial infection. Both of them can weaken the fish and make the fish easier infected by each other. This had been proven that high ectoparasites population in gills was the primary causation of bacterial infection. (Leong & Wong, 1988). The hooks, clamps, and suckers of the gill fluke can cause damage to the host's tissues leading to secondary bacterial infections (Katoch & Godra, 2014). This will lead to a serious economic loss if the affected fish was untreated in the earlier stage before the outbreak.

2.2 Diplectanid Monogeneans

Diplectanid monogeneans are one of the famous gill flukes. Monogenetic trematodes are highly pathogenic and obligatory parasites. They are commonly parasitic to the skin, fins, and gills of fish (Xu, 2014). Monogeneans have found more than 100 families from fishes of the world (Robert *et al.*, 2009). The most common genera in monogenean in marine fishes are *Diplectanum*, *Benedenia*, *Neobenedenia*, *Haliotrema* and *Pseudorhabdorsynochus*. In the family Deplectanid, *Pseudorhabdosynochus* sp. and *Diplectanum* sp. are relatively high host specificity in *Epinephelus* which is less than 5 mm in length (Schoelinck *et al.*, 2012; Nagasawa & Cruz-Lacierda, 2004). Both genera occur on the only single host and do not infect other fish (Leong *et al.*, 1999; Leong & Colorni, 2002; Justin & Poulin, 2008; Roumbedakis *et al.*, 2013).

Monogeneans are only found inhabiting the gill filaments and gill cavity of the fish host. It was an ideal site for monogeneans because both not only provides good water supply of blood and oxygen but a large surface area for attachment. The parasite also can prevent both physical and chemical damage under the protection of the operculum (Shaharom, 2012).

2.2.1 Description of Diplectanid Monogeneans

Monogenean's body shape may vary from round to elongate and dorso-ventrally flattened helminthes. This is due to the ability to stretch, bend and shorten their body (Justin & Poulin 2008). They can move rapidly in a leech-like manner if detached from gill filaments. Two pairs of eyes are present. The outermost layer of the parasite was called tegument formed by non-ciliated neodermis that cover by a glycocalyx and serve an osmoregulatory and excretory function.

Monogenean parasites only have a single opening which combining the both digestive and vascular system to form gastrovascular system (Klaus, 2001). The monogenean parasite has well-developed attachment structure that located at the posterior end called opishaptor. Opishaptor had sclerotinized structure in the form of 12-14 marginal hooks, but some species may absent. Each pair of anchors has transverse connecting bars, two pairs of hamuli, and two squamodiscs (Justin & Euzet, 2006; Erazo-Pagador & Cruz-Lacierda, 2010). The opishaptoral hooks may be able to penetrate the host tissue such as the epithelial to generate firm contact to the gill filaments. It also helps the parasite to feed on gill debris, dermal mucus, blood and tissue (Tucker, 1999; Robert *et al.*, 2009). The structure and measurement of the hold-fast organ may act as an identity of the parasite.

2.2.2 Life Cycle of Diplectanid Monogeneans (Figure 2.1)

Diplectanid monogeneans have a simple and direct life cycle, which they do not live on the intermediate host and attached to a single site on the host permanently. The reproduction rate of monogenean often accelerates when in poor environmental condition. Monogenetic flukes are a hermaphroditic parasite in the whole cycle which consist both male and female reproductive ability (Woo *et al.*, 2002). The reproductive organs such as the cirrus, accessory cirrus, and vagina act as an important taxonomic characters. They have a single testis, ovary, and follicular vitelline glands.

Depending upon the reproduction of the species, oviparous species usually produce sticky droplets and attach to the substrate while most of the viviparous monogeneans' eggs have a long and spiral filament which aid in attachment to substrates in the water or to the gill basket. The eggs will hatch and produce ciliated anteriorly, posteriorly and centrally larvae, the oncomiracidium (Erazo-Pagador & Cruz-Lacierda, 2010). They have straight line swimming behavior shown positive phototaxis that performs by oncomiracidia to seek a susceptible host in the water column (Erazo-Pagador & Cruz-Lacierda, 2010). Eyespots are also present to aid in locating the host. Since the fish is living closely together, the newly born larvae can attach quickly to the same host as their parent. It is also easy for the eggs to move direct or close contact between infected and susceptible hosts and spreading the infection at an exponential rate (Katoch & Godara, 2014). Within 2 to 6 days the eggs hatch into free-swimming larvae and die within 10 days if they do not find a host to parasitize.

Egg hatchling is temperature-dependent and temperature-controlled (Erazo-Pagador & Cruz-Lacierda, 2010). Eggs hatch at temperatures of 20 to 28°C within two to six days in the presence of fish mucus to produce the free swimming larval stage (FAO, 1996; Nagasawa & Cruz-Lacierda, 2004). At 20 to 28°C, the life span of the free swimming larvae is within 12 to 48 hours. However the ability for the larvae to reach their host will decrease after 4 to 6 hours (FAO, 1996). Initially, the gill parasites attach to the skin surface of the fish then migrate to their final attachment, gill filament and develop into adult stage in 14 to 21 days. Understanding the life cycles of the parasites is important for effective and successful treatment (Robert *et al.*, 2009).

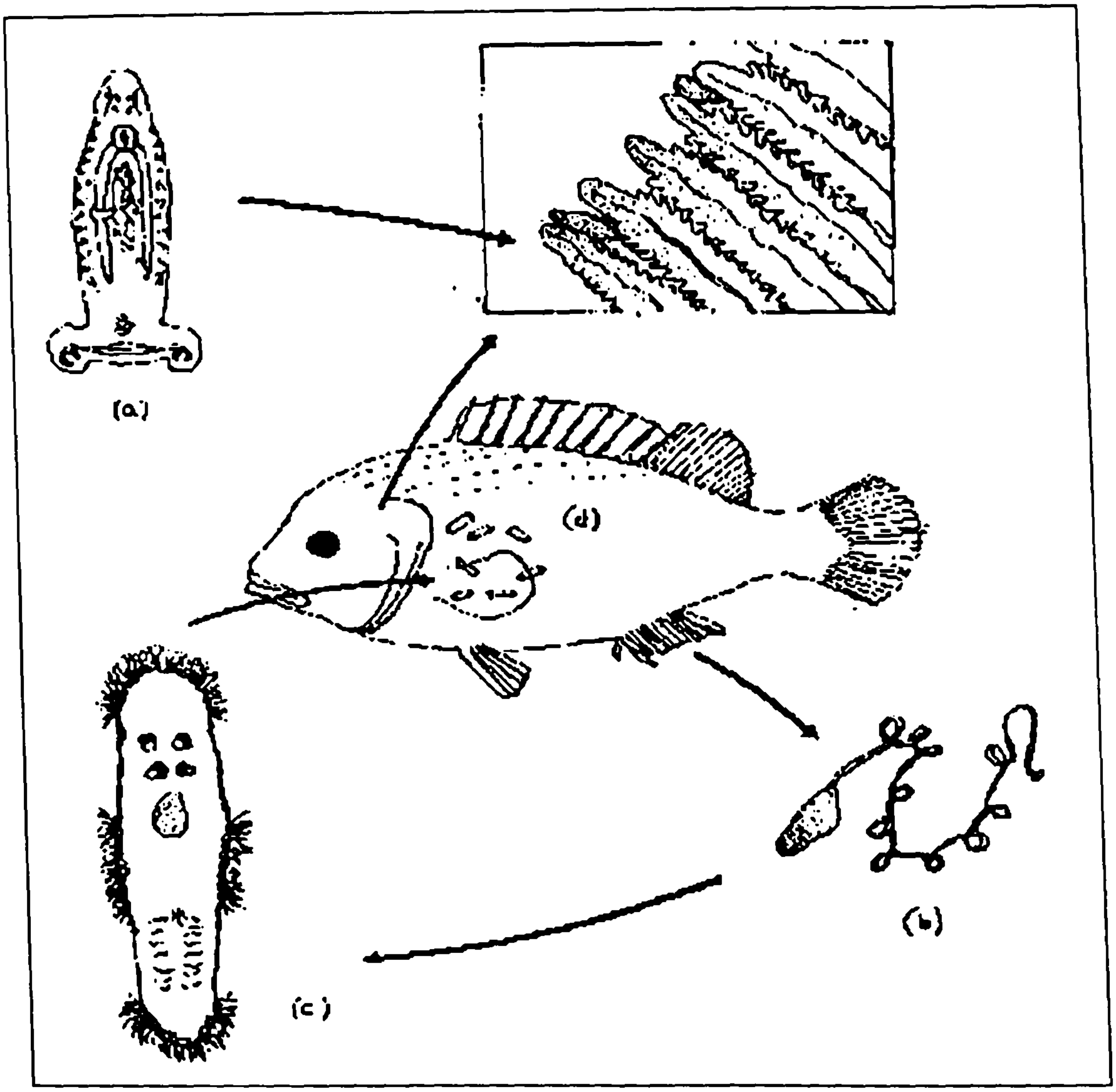

Figure 2.1 The life cycle of *Pseudorhabdosynochus lantauensis* parasitizing the gills of orange-spotted grouper, *Epinephelus coioides.*

(a) Mature fluke on gill lamellae

(b) Egg

(c) Free-swimming oncomiracidium

(d) Migrating post-oncomiracidium

(Source: Erazo-Pagador & Cruz-Lacierda, 2010)

2.3 Clinical Signs and Effects

Once the gill fluke disease occured, the fish show poor appetite, emaciated, dark in body coloration, lethargy and show abnormal swimming behavior (Katoch *et la.*, 2014). The fish also show clinical signs from suffocation such as gathering on the water surface where they gasp for air. It also causes hyperplasia which an abnormal increase in the number of cells in an organ and cause rapid opercula movement (Shaharom, 2012).

The affected gill filaments are pale and show characteristic mosaic appearance that covered with the mucous layer. It also results in anemia in the fish due to direct blood feeding of the parasite. When a large batch of mucus and parasites highly cover the gill lamellae it will cause respiratory failure, osmotic stress and lead to hypoxia. In some cases, grayish coloration of the tips of gill filaments and erosion of the distal parts of gill lamellae are seen. The damage is caused not only by feeding but also by the hooks and suckers used for attachment of the monogenean (Tucker, 2012). This causes damage and degenerations of epithelial cells in the gill filaments and rupture of the basement membrane at the lamella. Other than that, these cellular changes eventually interfere with the respiratory function of the gills and ending in the fish death.

The infected fish show paleness of muscle, liver and kidney. The value of haemoglobin content, protein in the serum, enzyme activities of lactate dehydrogenase, alkaline phosphatase, and glutamic pyruvic transaminase are lower than a normal fish. On the other hand, the level of creatinine and urea are higher in infected fish. Healthy grouper fingerlings showed 100% mortality within 72 hours of exposure to the gill fluke *Pseudorhabdosynochus* sp. at 5,000 monogeneans/10 fish (Inui, 2002). This is due to weak respiratory system and secondary infection caused by virus or bacteria. Therefore, if infected fish was not treated, it may cause serious problems.

2.4 Treatment

Since monogenean causes high mortality in fish, it needs therapeutic or prophylactic drugs to remove or kill this troublesome parasite to prevent further loss of income. There were some chemicals have been used to treat the flukes disease from some previous study (Table 2.1). There were only 2 types of drugs approved by Food and Drugs Administration (FDA) that use for food fish treatment shows in Table 2.2 which

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