Review

Etiology, pathogenesis and future prospects for developing improved vaccines against bluetongue virus: A Review

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Accepted 9 January, 2012

Bluetongue is a viral disease that primarily affects sheep, occasionally goats and deer and, very rarely, cattle. The disease is caused by an icosahedral, non-enveloped, double-stranded RNA (dsRNA) virus within the Orbivirus genus of the family Reoviridae. It is non-contagious and is only transmitted by insect vectors. BTV serotypes are known to occur in Africa, Asia, South America, North America, Middle East, India, and Australia generally between latitudes 35°S and 50°N. It occurs around the Mediterranean in summer, subsiding when temperatures drop in winter. The replication phase of the bluetongue virus (BTV) infection cycle is initiated when the virus core is delivered into the cytoplasm of a susceptible host cell. The 10 segments of the viral genome remain packaged within the core throughout the replication cycle, helping to prevent the activation of host defense mechanisms that would be caused by direct contact between the dsRNA and the host cell cytoplasm. This review presents comprehensive information on etiology, pathogenesis, prevention and control of the disease.

Key words: Bluetongue, orbivirus, pathogenesis, prevention.

INTRODUCTION

Bluetongue is an infectious noncontiguous virus disease of ruminants caused by bluetongue virus of genus orbivirus within the family reoviridae. It is transmitted by biting midges of the culicoides genus (Diptera: Ceratopogonidae). Among 1,400 species of midges only 20 culicoid species are known to be involved in transmission of bluetongue disease (Figure 1). In addition to biting midges, BTV has been isolated from some arthropods, for example, sheep ked (Melophagus ovinus) (Luedke et al., 1965) or some species of ticks (Stott et al., 1985; Bouwknecht et al., 2010) and mosquitoes (Brown et al., 1992). However, these are mechanical vectors with only a negligible role in disease epidemiology (Radostits et al., 1994). It can be directly transmitted from one animal to another through semen and transplacentally (Parsonson, 1990). Bluetongue can also be spread by live attenuated vaccines against BTV, or even by vaccines against other antigens contaminated with BTV (Wilbur et al., 1994; Evermann, 2008). To date 24 distinct internationally recognized serotypes (based on the lack of cross neutralization) of the virus have been identified. Cattle and goats are major hosts of the virus, but in these species infection is usually asymptomatic despite high virus levels, allowing the disease to circulate in the absence of any symptoms. Sheep and deer are usually the only species to exhibit symptoms of infection. The manifestations of bluetongue range from an unapparent to a fatal outcome depending on the serotype and strain of the virus, the species breed and age of the infected animal; older animals are generally more susceptible.
Bluetongue typically occurs when susceptible animal species are introduced into areas with circulating virulent BTV strains, or when virulent BTV strains extend their range to previously unexposed populations of ruminants (Zientara et al., 2010). The worldwide economic losses due to bluetongue have not been expressed in exact numbers, but the estimate is 3 billion US$ a year (Tabachnick, 1996). The losses are both direct (death, abortions, weight loss or reduced milk yield and meat efficiency) and, what is more important, indirect as a result of export restrictions for live animals, their semen and some products such as fetal bovine serum. The costs of preventive and control measures should also be taken into account. In cases of a wider spread of bluetongue, these measures could have a serious impact on the quantity of meat and animal products available for the consumer market; therefore, bluetongue is considered a potential biological weapon (Blancou and Pearson, 2003; Zendulkova and Pospisil, 2007).

**Occurrence**

Bluetongue was first recognized when European fine wool breeds of Merino sheep were imported into
The disease spread rapidly throughout Africa and subsequently to many other countries beyond the African continent. BTV occurs wherever there are species of Culicoides capable of transmitting it to animals. Historically BTV serotypes occur in Africa, Asia, South America, North America, Middle East, India, and Australia, generally between latitudes 35°S and 50°N (Figure 2). It occurs around the Mediterranean in summer, subsiding when temperatures drop in winter.

**Symptoms**

Symptoms of bluetongue disease are largely the result of damage to small blood vessels, and include oral ulceration, facial and pulmonary edema, vascular thromboses and necrosis of infected tissues (Erasmus, 1975) (Figure 1). In sheep, the onset of the disease is typically marked by fever that lasts approximately 5 to 7 days, after which distinctive lesions appear in the mouth, accompanied by excessive salivation. The tongue can also be severely affected, occasionally turning blue. In contrast to sheep, infected cattle experience prolonged viraemia, and infection during pregnancy can often cause teratogenic defects in calves and abortion of the fetus (De Clercq et al., 2008; Menzies et al., 2008; Vercauteren et al., 2008; Waldvogel et al., 1992).

**STRUCTURE OF VIRUS**

Bluetongue virus (density 1.337 g/cm³) is a non-enveloped virus with a genome of approximately 19,200 base pairs composed of ten linear segments of double-stranded RNA (dsRNA), containing 57% AU and 43% GC, with conserved 5' and 3' terminal sequences (GUUAAA at 5', and ACUUAC at 3' ends of the positive strand (Mertans et al., 1987; Mertans and Sangar, 1985). The 10 dsRNA segments are packaged within a triple layered icosahedral protein capsid (approx 90 nm in diameter) (Grimes et al., 1998; Huismans and Erasmus, 1981; Nason et al., 2004). The virus particle is composed of three shells (Figure 3). The inner shell is composed of 120 copies of VP3 (100 kDa) (Grimes et al., 1998) and contains minor amounts of 3 enzymatic proteins involved in transcription and replication, namely the RNA-dependent RNA polymerase VP1 (149 kDa), the RNA
capping enzyme VP4 (76 kDa) and the dsRNA helicase VP6 (36 kDa) that are located at the five-fold symmetry axis of the particle (Nason et al., 2004). The middle shell is composed of 780 copies of VP7 (38 kDa) that are arranged as 260 trimers (Nason et al., 2004; Roy, 1992). The outer shell is composed of two structural proteins VP2 (111 kDa) and VP5 (59 kDa). A total of 180 molecules of VP2 are arranged as 60 surface spikes which are responsible for attaching the virus to the cell surface, whereas 360 molecules of VP5 form 120 globular-shaped structures that facilitate cell-membrane penetration (Roy and Noad, 2006; Forzan et al., 2007; Roy, 2008). Non-structural proteins (NS1, NS2, NS3 and NS3A) probably participate in the control of BTV replication, maturation and export from the infected cell. The NS1 protein was found to play role in viral morphogenesis (Brookes et al., 1993; Owens et al., 2004). The NS2 protein is the major component of viral inclusion bodies (Brookes et al., 1993; Owens et al., 2004; Lymeropoulos et al., 2006; Schwartz-Cornil et al., 2008) and is also involved in recruitment of BTV mRNA for replication (Fukusho et al., 1989; Kar et al., 2007; Roy, 2008). The NS3 protein acts as a viroporin, which enhances permeability of the cytoplasmic membrane and thus facilitates virus release from mammalian or insect cells (Hyyat et al., 1991; Roy, 1992, 2008; Han and Harty, 2004). In addition, NS3 also allows BTV particles to leave host cells by a budding mechanism (Wirblich et al., 2006). This probably operates in insect cells where no cytopathic effect is induced by BTV (Schwartz-Cornil et al., 2008).

The segmented nature of the BTV genome allows for reassortment of ds-RNA segments if the host cell is concurrently infected by several different serotypes or strains (Oberst et al., 1987; Samal et al., 1987; Stott et al., 1987; Belyaev and Roy, 1993; Batten et al., 2008). The reassortment event plays an important role in the development of viral diversity (Carpi et al., 2010) and gives rise to changes in virulence and serological characteristics of the virus (Cowley and Gorman, 1989; Mertens et al., 1989; Nuttall et al., 1992; O’Hara et al., 1998; Batten et al., 2008).

BTV remains stable in the presence of proteins and can survive for years, for instance, in blood stored at 20°C (Anonymous, 2009a). It is sensitive to 3% NaOH, organic iodine complex, phenol and β-propiolactone (Radostits et al., 1994; Anonymous, 2009a).

**BTV life cycle**

BTV interacts with the target cell surface via VP2 timers which is then internalized in endosomes via a clathrin-dependent endocytosis pathway (Forzan et al., 2007). VP2 dissociates from the outer capsid layer in early endosomes. Acidification induces VP5 fusion with the endosomal membrane (Forzan et al., 2004), delivering the transcriptionally active core into the cytoplasm. BTV replicates in the cytoplasm of the infected cells. Within the core of BTV, the VP1 molecules transcribe positive...
sense ssRNA copies from each of the ten BTV genome segments (Boyee et al., 2004). These mRNA molecules are capped by the guanylyl-transferase and transmethylase activities of VP4 (Sutton et al., 2007) and leave the particles via channels situated at the fivefold axes of the core particle (Mertens and Diprose, 2004). The viral mRNA serve as templates for translation in viral proteins (Diprose et al., 2001). Viral positive RNA are directed to viral inclusion body (VIB) where the correct encapsidation of the different segments (nature and number) within the VP3 shell may involve interactions with the helicase VP6 (Stauber et al., 1997), the ssRNA binding NS2 protein (Kar et al., 2007), and the VP1 and VP4 proteins. VP1 then synthesizes the negative strand RNA to produce dsRNA (Boyee et al., 2004). Each dsRNA segment independently associates with a different transcription complex (VP1, VP4 and VP6) located at the inner side of VP3 along a fivefold axis (Nason et al., 2004). The VP3 subcores which are relatively fragile (Roy, 1992) serve as a scaffold for the addition of VP7 trimers, giving rise to more rigid and stable cores. The outer capsid proteins VP2 and VP5 appear to be added to the progeny core particle surface at the periphery of the VIB as they enter the host cell cytoplasm. Mature progeny virus particles are transported within the cytoplasm on microtubules involving VP2/vimentin interactions (Bhattacharya et al., 2007).

Release of virions from the infected cell occurs via cell membrane destabilization mediated by the NS3 viroprotein activity (Han and Harty, 2004), in some cases via budding, or as a result of cell death and lysis (Figure 4).

**Overwintering**

The survival of virus from one “vector season” to the next is called “overwintering”, but the mechanism involved is still poorly understood. However, BTV can survive in the absence of adult vectors for nine to 12 months of cold weather in an infected host with no detectable viraemia, disease or sero-conversion (Taylor and Mellor, 1994; Takamatsu et al., 2003; Osmani et al., 2006; Wilson et al., 2007). One way in which overwintering may be achieved is by the infection of adult vectors (Wilson et al., 2008). Although the average life span of these is usually ten to 20 days (Mellor et al., 2000), they can occasionally live for up to three months (Lysyk and Danyk, 2007). This suggests that under favourable conditions some biting midges can live long enough to survive the period between two vector seasons (Wilson et al., 2008). Possibilities for BTV to survive at different stages of the *Culicoides* life cycle have also been investigated. In some instances, the virus could overwinter in cattle owing to
prolonged BTV viraemia, which can occasionally last up to 100 days (Sellers and Taylor, 1980), or due to latent BTV infection (Luedke et al., 1977). Another mechanism suggested for BTV overwintering is transplacental infection (De Clercq et al., 2008; Menzies et al., 2008; Backx et al., 2009; Darpe et al., 2009; Leverin et al., 2010; Santmier-Berends et al., 2010). Pregnancy in cattle is long enough for BTV to survive during a period free of competent insect vectors (Wilson et al., 2008). Mechanical vectors may also be involved in virus overwintering; BTV has been isolated from the sheep ked (Luedke et al., 1965) and some tick species (Stott et al., 1985; Bouwknegt et al., 2010), which are arthropod species living much longer than Culicoides midges. In addition, the trans-stadial passage found in hard ticks and trans-ovarial passage in soft ticks suggests their role in virus transmission (Bouwknegt et al., 2010). Mechanical vectors should therefore be regarded as potential reservoirs for BTV (Wilson et al., 2008; Bouwknegt et al., 2010).

**BTV pathogenesis**

After introduction through the bite of an infected midge, the virus is transported by the host dendritic cells from the skin to the local lymph nodes (Hemati et al., 2009), the sites of initial virus replication (MacLachlan, 2004). Subsequently, it spreads to the blood circulation inducing a primary viraemia which seeds secondary organs, that is, lymph nodes, spleen and lungs (Barratt-Boyes and MacLachlan, 1994; Sanchez-Cordon et al., 2010). The virus replicates in vascular endothelial cells, macrophages and lymphocytes (MacLachlan et al., 1990, 2009; Barratt-Boyes and MacLachlan, 1994; MacLachlan, 2004; Drew et al., 2010a). In early viraemia virus is associated with all blood elements, while at later stages of viraemia it exclusively associates with erythrocytes (MacLachlan et al., 1990, 2009; MacLachlan, 2004). Virus particles appear to be sequestered in invaginations of the erythrocyte membrane (Brewer and MacLachlan, 1994; MacLachlan, 2004), allowing prolonged viraemia in the presence of neutralizing antibodies (Richards et al., 1988; Brewer and MacLachlan, 1994). Free virus in low titres is found in blood plasma only at the initial stages of infection (MacLachlan et al., 1990; MacLachlan, 1994; Barratt-Boyes and MacLachlan, 1994).

Infection with BTV results in cell necrosis and apoptosis (Barratt-Boyes et al., 1992; DeMaula et al., 2001; Mortola et al., 2004) and, by activating the p38MAP kinase, the virus increases vascular permeability (Chiang et al., 2006; Drew et al., 2010a). In addition, it induces the production of TNFα, IL-1, IL-8, IL-6, IFN-I and cyclooxygenase-2, and enhances plasma concentration of prostacyclin and thromboxane, which frequently leads to an excessive inflammatory response and subsequent damage to the cells and tissues of the infected animal (MacLachlan and Thompson, 1985; DeMaula et al., 2001, 2002; Schwartz-Cornil et al., 2008; Drew et al., 2010a). The pathogenesis of bluetongue is characterised by injury to small blood vessels in target tissue, resulting in vascular occlusion and tissue infarction. Virus-induced vasoactive mediators produced by thrombocytes, dendritic cells, macrophages and BTV-infected endothelial cells increase damage to the endothelium, interfere with its function and increase vascular permeability; this leads to the development of oedema and effusions (MacLachlan et al., 2009; Drew et al., 2010a).

**Viraemia and immune response**

Viraemia in infected animals has a prolonged course, but is not persistent (Barratt-Boyes and MacLachlan, 1994; Bonneau et al., 2002; Melville et al., 2004). Its duration depends on the longevity of erythrocytes to which virus is bound, in contrast to the other blood cells, even at the late stage of infection (MacLachlan et al., 2009). It is also related to the species and breed of the infected animal. Viraemia lasts 14 to 54 days in sheep and 19 to 54 days in goats (Barzilai and Tadmor, 1971; Luedke and Anakwenze, 1972; Koumbati et al., 1999). In cattle, viraemia may last as long as 60 or, even 100 days (Sellers and Taylor, 1980), which makes this animal an important host, from the epidemiological point of view.

The infected animals react to BTV with interferon production and humoral and cell-mediated immune responses (MacLachlan and Thomson, 1985; MacLachlan, 1994). Serotype-specific neutralising antibodies against the VP2 protein confer protection against homologous strain reinfection (Cowley and Gorman, 1987; Hassan and Roy, 1999; Roy, 1992; Schwartz-Cornil et al., 2008). Neutralising antibodies are also induced, to a lesser degree, by the VP5 protein (Roy, 1992; Lobato et al., 1997). The sera of infected ruminants also contain serogroup-specific antibodies induced by the VP7 protein, as well as antibodies against other structural and non-structural proteins (MacLachlan et al., 1987; Richards et al., 1988; MacLachlan, 2004). The cell-mediated immune response to BTV can probably reduce the spread of virus in the organism early after infection, but cannot eliminate the virus completely (MacLachlan, 1994; Barratt-Boyes et al., 1995). By producing a cytotoxic effect in infected cells, CD8+ T-lymphocytes play the most important role (MacLachlan,
PREVENTION AND CONTROL

Symptomatic therapy includes gentle handling of affected animals, their stabling and, if indicated, administration of non-steroidal antiphlogistic drugs (Radostits et al., 1994; Tweedle and Mellor, 2002). An immediate ban on animal import from countries with bluetongue is the priority measure, followed by the monitoring of farms raising domestic ruminants which include clinical examination and serological and virological testing, and a monitoring of insect vectors. Prophylactic immunisation and the removal of vectors or prevention of vector attacks can also be used.

Prophylactic immunisation

Vaccination can prevent clinical bluetongue or at least mitigate its course by interrupting the BTV cycle in the environment; it thus reduces the economic losses due to animal infection and makes transfer and trading of animals from BTV enzootic regions possible (Savini et al., 2008; Bhanuprakash et al., 2009; Caporale and Giovannini, 2010). Bluetongue vaccines are serotype-specific (Bhanuprakash et al., 2009) and therefore, before use in a given area, the serotypes present in the environment should be taken into account.

Vaccination

The most widely discussed vaccine options for the control of bluetongue are live attenuated, inactivated and recombinant vaccines.

Attenuated viral vaccine

Attenuated virus vaccines are cheap, easy to produce and are administered in a single dose. They are very effective in controlling clinical outbreaks of bluetongue in areas of endemic disease and in the face of outbreaks. They replicate in sheep without causing significant clinical effects and provide protection against challenge with virulent virus of the same serotype. Animals vaccinated with the attenuated vaccine produce a long lived humoral antibody response, possibly lasting for the life of the animal.

An early report of live attenuated bluetongue vaccines came from South Africa (Theiler, 1906). Subsequently, South African scientists developed the first egg-adapted attenuated strains. This work led to the availability of attenuated virus vaccines for 15 different serotypes, which played a major part in control of the disease not only in South Africa but also in many other countries (Alexander and Haig, 1951). However, similar, live attenuated vaccines have also been developed in various countries in response to endemic serotypes or a particular outbreak (Alexander and Haig, 1951; Hunter and Modumo, 2001; Lacetera and Ronchi, 2004; Veronesi et al., 2005; Savini et al., 2008).

However, the disadvantages of attenuated BTV vaccines (Schultz and Delay, 1995; Young and Cordy, 1964; Osburn et al., 1971; Ferrari et al., 2005; Stott et al., 1987) are:

1) Risk of reassortment with virulent wild viruses which potentially could give rise to new virulent strains.
2) Potential for reversion to virulence both in the vertebrate host and in vector insects.
3) Attenuated BTV can cross the placenta and pregnant ruminants vaccinated with attenuated vaccines may suffer fetal loss.
4) Existing vaccines are designed for sheep; there is little data on their safety and effectiveness in other species.

Inactivated whole virus vaccine

They offer significant advantages over attenuated vaccines because absence of replicating virus eliminates concerns about virarmia, vector transmission and reversion to virulence. It also eliminates the danger of fetal infection, often reported for attenuated bluetongue vaccines, and eliminates the possibility of viral reassortment. The use of inactivated vaccines also allows a rapid response to newly emerging serotypes. Once a new serotype has been isolated from the field, it can be rapidly propagated to produce a homologous approaches which would take much longer to sequence, clone and produce a suitable treating BTV with β-propionalactone (Parker et al., 1975; Savini et al, 2007), gamma radiation (Campbell, 1982) or binary ethylenimine (Schultz and Delay, 1995; Berry et al., 1982; Ramakrishnan et al., 2006). Inactivated vaccines based on these experiments are commercially available and have demonstrated good immunogenicity and safety (Savini et al., 2008). However, inactivated vaccines are more expensive to produce than attenuated vaccines and also require at least two doses with an adjuvant to generate a protective immune response. Antibodies to inactivated vaccines are short lived, giving short term protection.
Recombinant vaccines

Recombinant vaccines such as recombinant virus-like particles (VLP) or single BTV antigens are safe and have been shown to be efficacious (Noad and Roy, 2003; Roy and Noad, 2008; Harper et al., 2006; Barr and Tamms, 2007; Franco and Harper, 2005; Harper et al., 2004). VLP vaccines are the only vaccines that have been subjected to a number of clinical trials in different countries. They require two inoculations of low doses for long lasting protection. Due to recent advances in the manufacturing of insect cell cultures, VLP vaccines could be very cost effective.

New – generation vaccines

Vaccination has been a successful methodology to combat diseases in man and livestock. Most of the current viral vaccines are prepared using attenuated or inactivated virus. Control of Bluetongue (the disease) is particularly difficult due to the multiple serotypes of the virus. In addition, the viral genome is made up of 10 segments allowing exchanging the genes randomly between different viruses. This may cause generation of infectious virus with mixed gene. Recent recombinant DNA technology has provided novel approaches to developing intrinsically safe vaccines, these vaccines are not yet commercially available. This technology offers substantial advantages both in terms of safety and the potential of developing a marker vaccine. The latter could be used as a prophylaxis in areas at risk, without endangering the “free” status of the region. An accompanying serological test would allow the distinction between vaccinated and infected animals. DNA recombinant technology involves the synthesis of immunogenic proteins and particles that elicit highly protective immune responses. Successful vaccine development requires systems where the engineered products mimic the authentic proteins, not just in terms of their primary amino acid sequences but specifically in terms of their three dimensional structures, that is, the products must be as authentic as possible.

In recent years insect (caterpillar) specific baculoviruses have received considerable attention as vectors for the high-level synthesis of foreign proteins. Protein engineering systems were utilized to synthesize individual bluetongue virus proteins and core-(single coat) and viral-like (double coat) multiprotein structures (CLPs, VLPs). These 16 engineered particles essentially mimic the virus particles, but do not contain any genetic materials (French et al., 1990; Possee et al., 1999; French and Roy, 1990; Hewat et al., 1992; Hewat et al., 1994; Loudon et al., 1991) (Figure 5).

VIPs afford long-lasting, type-specific protection from virulent BTV challenge. In addition, mixtures of VIPs for
two different serotypes confer complete protection against both vaccine serotypes and partial protection against a related (based on the amino-acid sequence of VP2) non-vaccine serotype. Therefore, VIPs represent a valid approach for BTV vaccination.

**Reverse genetics and future vaccines**

Traditional live vaccines for BTV rely on the attenuation of virus by passage in eggs or sheep. The recent development of a reverse genetics system for BTV makes possible the rational design of attenuated vaccines. Infectious BTV is produced entirely from DNA clones by generating one transcript *in vitro* for each genome segment, and using these transcripts to transfect permissive cells (Harper et al., 2006). This system allows the introduction of any mutation into the genome of BTV, as long as the resulting virus is viable. The ability to test the virulence of BTV mutants in the ruminant host will allow the identification of the pathogenicity determinants of BTV, and these results can be used to inform the design of vaccine strains with multiple attenuating mutations. Reverse genetics data and the formation of BTV VIPs have confirmed that outer capsid proteins from phylogenetically diverse serotypes can assemble on the conserved core proteins to create viable BTV strains (Loudon et al., 1991; Boyce et al., 2008). This observation suggests that it will be possible to use a defined attenuated genetic background and introduce the antigenically important outer capsid proteins from the serotypes of interest. Reverse genetics also provides a basis for the development of disabled infectious single cycle (DISC) vaccines for BTV (Figure 6), which allows the virus to infect the vaccinated animal, but stops it from completing a replication cycle. The resulting aborted infection allows the expression of viral proteins at natural sites of infection without the production of infectious virus or disease in the animal, and can be considered to be an
extreme form of attenuation. A DISC vaccines strain would exhibit many of the safety features of inactivated vaccines, while preserving the expression of viral proteins at the natural sites of infection, as observed with live vaccines. DISC vaccines and colon bias (Roner and Joklik, 2001; Coleman et al., 2008) vaccines for BTV represent an exciting future possibility, as they should allow increased safety with even better immunogenicity.

CONCLUDING REMARKS

The epidemiological map of BTV infections in Europe illustrates the risk to the entire world of emerging microbial diseases that were previously confined to specific geographic areas, spreading due to global warming and increased trade. It also reveals that many questions remain regarding the physio-pathological mechanisms of the BTV induced thrombo-haemorrhagic disease, the species/breeds/individual genetic bases of sensitivity to BT disease, the molecular basis of BTV virulence, the insect and mammalian reservoirs, and the immune effectors involved in cross protective immunity. The control of bluetongue disease through timely and relevant vaccination is feasible. However, although current vaccines are effective, they have significant drawbacks that are likely to increase as the demand for vaccination grows. The speed of scale up to a newly emerged serotype, the uncertain nature of natural attenuation and the safety issues associated with virus inactivation all suggest that newer approaches are needed. The new generation of vaccines described above offers a rapid route from DNA to vaccine as well as a safe product without loss of a robust immune response.

ACKNOWLEDGEMENTS

The author acknowledge Professor V. Uma, Department of Chemistry, Nizam College, for providing technical assistance in carrying out this work and Dr. Y. Vishwa Kiran for the critical review of the manuscript.

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