https://doi.org/10.22319/rmcp.v13i3.5564

Review

Diagnosis, prevention and control of diseases caused by *Chlamydia* in small ruminants. Review

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Abstract:

The species that make up the genus *Chlamydia* affect a wide range of animal hosts, causing various pathologies. *Chlamydia abortus* (*C. abortus*), *Chlamydia psittaci* (*C. psittaci*) and *Chlamydia pecorum* (*C. pecorum*) are the most clinically relevant in small ruminants worldwide, since they have been related to reproductive, ocular and digestive tract problems respectively; two of these (*C. abortus* and *C. psittaci*) represent a potential zoonotic risk to humans. The diagnosis of infections by organisms of this genus is complicated; since, in most cases, there are no clinical signs that indicate the presence of the agent in affected animals. Currently, in European countries, the prevention and control mainly of *C. abortus* is carried out through the administration of commercial attenuated immunogens; however, their use has not shown satisfactory results in the protection of susceptible animals. Therefore, the implementation of new immunization options based on the utilization of recombinant proteins is the line of research that is currently taking the most prominence. Additionally, the use of proteins with immunogenic potential could be important tools for the diagnosis, prevention and control of these pathogens. Due to this, the present review focused on recapitulating the most current studies focused on the experimental use of different immunogenic proteins of *Chlamydia* spp. used worldwide, highlighting their innovation and results obtained in experimental models.

Key words: *Chlamydia*, Diagnosis, PCR, Recombinant Proteins, Vaccines.

Received: 28/11/2019

Accepted: 02/12/2020

Introduction

The bacteria of the genus *Chlamydia* are Gram-negative, obligate intracellular organisms that are characterized by sharing a unique biphasic development cycle, they have two morphological structures called: elementary body (EB), which is the infective form, and the reticulate body (RB), form of the metabolically active bacterium[(1)]. These bacteria cause a wide range of diseases in different animal host and man[(1,2)]. Within the genus, a total of twelve species have been reported[(3)]: *C. trachomatis*, *C. muridarum*, *C. suis*, *C. psittaci*, *C. abortus*, *C. caviae*, *C. felis*, *C. pneumoniae*, *C. pecorum*, *C. avium*, *C. gallinacea*, *C. poikilothermis* and four species candidates: *C. ibidis*, *C. serpentis*, *C. corallus* and *C. sanzinia*[(4-6)].

Some of these species tend to affect production animals; in addition, they represent a potential zoonotic risk to humans[(7)]. The main species of the genus identified in these animal species are: *C. abortus*, *C. psittaci* and *C. pecorum*; in addition, the pathologies associated with them have been widely documented[(1,2,8)].

The pathologies related to these organisms are diverse, among which the following stand out: abortions, keratoconjunctivitis and problems in the digestive tract[(2,9)]; however, *C. abortus* is the most important in livestock production, generating greater losses in flocks than due to the presence of *C. psittaci* and *C. pecorum*[(1,2)].

Due to the importance for public and animal health that these organisms represent, this review focused on compiling the most recent studies on the development of diagnostic tests, treatment and control of infections caused by these bacterial species, where studies focused on the production of recombinant proteins stand out, which have been the subject of study in recent years.

Species of the genus *Chlamydia* affecting small ruminants

Chlamydia abortus

Causative agent of Ovine Enzootic Abortion (OEA), a disease that is widely distributed worldwide, which causes economic losses in countries that are engaged in livestock activity. The disease causes abortion in pregnant ewes in the last third of gestation or, in some cases, the birth of weak lambs that do not exceed 48 h of life[1,2]. It is currently considered the most important pathology of chlamydial origin; since it represents a potential occupational zoonotic risk and for pregnant women who are in contact with infected animals[2]. In this order, different reports have been made in which, in addition to causing abortions, it causes other conditions, such as: febrile illness, development of disseminated intravascular coagulation, acute renal failure and pulmonary edema[10], septicemia and important lesions in the liver, kidney and heart; it is worth mentioning that these pathologies occurred after the abortion[11]. It is also described as a causative agent of pelvic inflammatory disease[12]. In Mexico, the prevalence of antibodies against *C. abortus* was reported in exposed risk groups (workers and veterinarians) who were in contact with flocks with a history of abortion[13]. Finally, it has also been reported as a causative agent of pneumonia problems[14], thus demonstrating the zoonotic potential of this bacterial species.

Chlamydia psittaci

Bacterium of avian origin that causes psittacosis in birds and with proven potential zoonotic risk. In the last five years, different studies have been carried out, evidencing the risk that this bacterial species represents for humans, mainly due to contact with infected birds[2]. In the first instance causing psittacosis, ornithosis[2] or atypical pneumonia[15]. It has also been reported as a causative agent of genital infections in women[16]. It has been identified in patients with respiratory diseases; for example, pneumonia in farmers who worked with infected animals[17]. Similarly, it has been linked to pneumonia, pertussis and conjunctivitis[18]. Not least, associating the possible risk factors involved in the contagion of psittacosis in people who handle birds[19,20]. Recently, it was reported as a causative agent in an outbreak related to severe respiratory disease among workers of poultry slaughter plants in the USA[21]. There are currently no reports of contagion of *C. psittaci* from mammals to humans.

Chlamydia pecorum

This bacterium lodges naturally in the digestive tract and causes enteritis; in most cases, it occurs subclinically, thus avoiding the timely detection of the disease[22]. *C. pecorum* has also been associated with other diseases, among them: arthritis, keratoconjunctivitis, encephalomyelitis, infertility, pneumonia and mastitis, and causing economic losses in production units[22]. Despite the wide variety of pathologies with which this bacterial agent has been related, the zoonotic potential of this species of *Chlamydia* is still unknown[23].

Transmission

It has been widely documented that the way in which these organisms are mostly transmitted is through the oronasal route[1]. *C. abortus* is excreted by infected ewes through vaginal fluids or placental remains, which contaminate water or food, the entry to susceptible animals is through the intake of these[2].

C. psittaci is usually contained in the feces of birds and small ruminants, therefore, the main route of transmission of this pathogen is through the inhalation of aerosols contaminated by feces, in feeders or outdoor areas[7].

Transmission of *C. pecorum* is thought to take place by the oral-fecal route or by ingestion or inhalation of bacteria contained in secretions of infected animals. Some studies have suggested transmission as a result of factors such as mutual grooming, inhalation and overcrowding[22].

Diagnosis

Due to the variety of clinical pictures, animal hosts and since these agents are often diagnosed in combination with other infectious agents, a definitive diagnosis usually requires laboratory tests in most cases[24]. The diagnosis of diseases of chlamydial origin is complicated and requires complex methodologies that require highly trained personnel to be able to carry out an ideal diagnosis[24]. To make the diagnosis of *Chlamydia* species, the samples by choice are swabs (vaginal, conjunctival and/or rectal) preserved in a special transport medium for *Chlamydia* spp., sucrose-phosphate-glutamate (SPG)[24,25]; on the other hand, the diagnosis can be made by indirect (ELISA) or direct methods (cell culture and PCR)[24].

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is an indirect diagnostic test that detects antibodies in serum of affected individuals against specific antibodies of bacteria of this genus[2,24], there are currently commercially available ELISA tests, among the disadvantages of this type of tests are cross-reactions between species (*C. abortus* and *C. pecorum*), which makes specific diagnosis difficult[2]. The assays that evaluate different antigens for the detection of species of *Chlamydia* spp. have been diverse. First, evaluating fragments of the MOMP through an indirect ELISA test (rOMP91B iELISA), which showed a sensitivity and specificity of 84.2 and 98.5 % respectively; the study showed that the indirect ELISA test was better at differentiating animals infected with *C. abortus* and *C. pecorum*[26]. Later, another study evaluated different recombinant antigens, all of these were identified from the Polymorphic Outer Membrane Protein or POMP90. Of the 11 fractions identified, OMP90-3 and OMP90-4 were the most effective, showing a sensitivity of 95.7 and 94.3 % respectively and a specificity of 100 % for both. The findings of the study revealed that the ELISA test with the fragment rOMP90-4 was more sensitive than that of rOMP90-3, since it identified more positive samples for OEA and, in addition, both were superior to the complement fixation test (CFT)[27]. Additionally, and in a complementary way to these studies, a study was carried out in which four experimental ELISA tests based on complete EB of *C. abortus* (EB), a preparation from the outer membrane of complete bacteria (SolPr) and two recombinant fragments of POMP90 (rOMP90-3 and rOMP90-4), were evaluated against three commercial tests, the CHEKIT1 *Chlamydophila abortus* test, Pourquier1 ELISA *Chlamydophila abortus* and ImmunoComb Ovine *Chlamydophila* Antibody. The results during the test showed that the commercial ELISA test InmunoComb obtained the highest sensitivity (98.4 %) compared to the others; however, the specificity determined (65.4 %) was lower than all tests evaluated. The results at the end of the study determined that, of the eight ELISA tests evaluated, the test that offers the best results in terms of sensitivity and specificity was the ELISA test based on the recombinant fragment rOMP90-3, with values of 96.8 % and 100 % respectively, this study showed that this experimental ELISA test can be an adequate alternative for the serological diagnosis of OEA[28].

A study carried out in 2018 adds to these results, which compared three commercial tests (IDvet, MVD-Enfer and LSI) for the detection of antibodies against *C. abortus* in ewes, vaccinated animals were evaluated for different periods of time to measure the production of antibodies between animals that aborted and animals that reached term, the results revealed that the most sensitive test was the LSI (94.74 %) followed by MVD-Enfer (78.95 %) and finally IDvet (73.68 %), all three kits detected high levels of antibodies in ewes that aborted compared to those that had lambs without complications. The most sensitive test in this study is based on the identification of chlamydial lipopolysaccharide (LPS), which shows cross-

reaction with all species of the genus *Chlamydia*, it is determined suitable for identifying ewes infected with any species of *Chlamydia*, but it is not considered specific for *C. abortus*[29]. Finally, a study revealed the disadvantages of this type of commercial tests in terms of cross-reactions between *C. abortus* and *C. pecorum*, carrying out the evaluation in different flocks, serological tests revealed a low seropositivity of *C. abortus* using an ELISA test based on peptides (1.2 %) in Australian ewes and a moderate seropositivity in a Swiss flock with a clinical history of abortion associated with *C. abortus* (26.9 %). Using CFT and ELISA tests, seropositivity was significantly higher, suggesting cross-reactivity between these two species. Additionally, using a real-time PCR test to detect DNA of *C. pecorum* in Australian animals seropositive for *Chlamydia* spp., it was concluded that the seropositivity of *Chlamydia* may be related to cross-reactivity with endemic infections of *C. pecorum*[30]. Due to the disadvantages shown by this type of tests, it is advisable to complement them with a more specific one such as PCR tests[24].

Cell culture

Due to their obligate intracellular nature, these bacteria require live media for their isolation; due to this, cell culture (McCoy cells by choice) is currently used for this purpose[24], until a few years ago, this technique was considered the gold standard for the diagnosis of *Chlamydia* spp.[1]; however, the development of new methodologies, such as nucleic acid amplification (PCR and sequencing), employed to improve diagnosis are currently considered the gold standard for diagnosing *Chlamydia* spp. infections[8]. Other tests such as polymerase chain reaction, which is a much more specific technique for the detection and typing of species of *Chlamydia* spp., is used much more frequently because it has a greater sensitivity and specificity compared to cell culture[2].

Polymerase chain reaction test

This test detects specific DNA of any organism; therefore, it is a much more sensitive and specific test than cell culture when identifying the genus and species involved in the affected individuals[24]. In the last 15 yr, the use of this technique has become very relevant, and its different variants reveal better results compared to those mentioned above, such as: i) processing a greater number of samples, ii) less time to obtain results, iii) the use of different types of samples for diagnosis, iv) organisms do not have to be one hundred percent viable and v) greater sensitivity and specificity. Since its use in veterinary diagnostic laboratories was implemented, the genes used to identify these bacterial agents have been different: major

outer membrane protein (MOMP), polymorphic membrane proteins (Pmps), 16S and 23S[24]. PCR methodologies for the specific detection of *Chlamydia* species are varied, to mention a few: The PCR test "Touchdown enzyme time release" to amplify different DNA sequences in the variable regions of the spacer rRNA genes 16S and 16S-23S specific for the identification of species of this bacterial genus; for example, *Chlamydia trachomatis*, *Chlamydia pneumoniae* and *Chlamydia psittaci*[31]. Another variant of this test, the PCR-RFLP test that identifies in the first instance the presence of the *omp2* gene specific to the family Chlamydiaceae and later, by digestion with the restriction enzyme *AluI*, has the ability to identify a total of nine species of the genus *Chlamydia*, among these, *C. abortus*, *C. pecorum*[32] and *C. psittaci*[33]. Additionally, a variant focused on the 16S gene specific to the family Chlamydiaceae but using a real-time PCR test demonstrated high specificity when evaluating samples of different *Chlamydia* species against other bacterial genera[34]; additionally, this variant can also be used for the specific identification of *Chlamydia* species using specific primers for each one[35]. Later, a multiplex PCR test was developed for the identification of *C. abortus*, *C. pecorum* and *Coxiella burnetii*, involved as causative agents of abortion, this test, unlike the aforementioned ones, helps to simultaneously identify the three species, this test proved to be highly specific and rapid for the detection of these bacterial agents[36].

Treatment

Antibiotics

For the treatment of pathologies of chlamydial origin, antibiotics are the drugs of choice, since bacterins, in the case of OEA, are only available in some European countries and the costs required for their implementation are very high[2,7]. The administration of tetracycline, penicillin and chloramphenicol for the treatment of infections caused by these bacterial agents has been shown to inhibit the growth of these organisms[24], it is important to emphasize that the use of antibiotics should be in a controlled manner to minimize the development of resistance by the pathogen. Although antibiotics serve to reduce losses due to these pathogens, these types of treatments do not eliminate bacteria; since affected animals continue to shed the organisms; therefore, their prophylactic use is not recommended[37]. In European countries, in addition to the use of antibiotics, the administration of bacterins is also implemented in the case of OEA to prevent and control the disease in flocks with susceptible animals, this is because it is the only disease of chlamydial origin for which there are commercially available bacterins[2].

Immunogens

In the past century, immunogens intended for the treatment and prevention of diseases worldwide have been one of the greatest achievements of public health; in this area, between 2 and 3 million lives are saved due to the implementation of these health measures[38]. In the veterinary field, technological advances in the development of immunogens for disease control have had an important enhancement in the last 25 yr, from the use of complete bacteria, whether alive or dead, to the use of DNA immunogens, which offer safer measures for both the animal and the veterinarian who administers them[39].

In the last 70 yr, studies for the development of immunogens to combat diseases of chlamydial origin in animal species, mainly livestock (sheep, goats, cattle and pigs), focus on preventing economic losses in production units; however, the main objective is to preserve human health due to the zoonoses that some of these represent. Vaccine trials against *Chlamydia* spp. have increased in the last 10 yr, in these studies, the protein most used in the challenges against *Chlamydia* spp. has been the MOMP[40]. Additionally, studies focused on the search for specific antigens of this surface protein have been carried out for a differentiation between species[41]. Later, different types of antigens have been used in the development of immunogens against chlamydial agents, the first tests traditionally used the EB, which were inactivated by treatments with ultraviolet light or live (attenuated) fixed with formalin. Later, in the mid-1990s, approaches in the use of other antigens for the development of subunit vaccines, such as recombinant proteins, synthetic peptides, expression vectors and DNA, began to be used for the challenge in murine models mainly[40].

In the case of *C. pecorum*, there are only two trials of vaccines against this agent, which have been challenged in murine models, although the results revealed an immune response in animals, these should be treated with caution because clinical cases of abortions are rarely related to *C. pecorum*[42].

Finally, although there are commercially available vaccines for the control and prevention of *C. abortus*, it is well documented that in the case of the live attenuated 1B *C. abortus* vaccine, it has the potential to reactivate and cause the disease in immunized animals[43-45].

Of the total vaccine challenges against *Chlamydia* spp., only 5 % have been focused on *C. abortus*, evaluated in mice, cows, ewes and guinea pigs[40], the studies evaluate some proteins, mainly Pmps, looking for different variations or mutations that can serve as key points for the prevention of OEA[46].

Subunit vaccines, new technologies for the development of serodiagnostic tests and vaccine prototypes

To date, different studies that have identified these possible candidates, both for the diagnosis and for the development of vaccines[2], have been developed. Immunoreactive proteins, expressed in infected animals, have been proposed as new candidates as marker antigens for the diagnosis and use of different virulence genes that can be used for the development of prototypes of subunit vaccines for the prevention and control of diseases caused by species of *Chlamydia* spp.[47]. In the case of *C. abortus*, several studies have been carried out, evaluating different proteins. A study evaluated the humoral immune response triggered by some surface proteins (MOMP, MIP, Pmp13G) and associated with virulence (CPAF, TARP, SINC), this study showed that ewes that aborted showed a strong antibody response to surface antigens. Additionally, they identified that the most specific antigen for the serodiagnosis of human infections by *C. abortus* was Pmp13G; this protein did not show cross-reactivity with other species of *Chlamydia* spp. that affect humans[47].

Regarding the proteins currently used for the development of vaccine prototypes, studies have focused on three proteins that play a major role in the development cycle of the bacterium[40]. Other proteins of this bacterial genus used as antigens in vaccine trials are the membrane surface proteins of *Chlamydia* spp., which have been shown to have highly conserved regions[48]. Heat shock proteins (HSPs) and chlamydia protease-like activity factor (CPAF) proteins have also been considered as suitable candidates for the development of immunogens for the control and prevention of diseases of chlamydial origin, these have been shown to cause a strong inflammatory response in the host[49]. Another study evaluates three different types of vaccines: DNA vaccine, phage vaccine (*OmpA*) and a commercial live attenuated, freeze-dried vaccine based on the strain 1B of *Chlamydia abortus*. Although the phage vaccine offers good results, it does not exceed that offered by the commercial vaccine; however, the study concludes that this novel vaccine administration system offers advantages that far outweigh commercial vaccines, such as: handling, more efficient safety and relatively cheaper production[50]. Other tests evaluated a combination with the polysaccharide *Lycium barbarum* (LBP3a), the results demonstrated good protection in mice challenged with *C. abortus* using a polysaccharide LBP3a combined with a DNA vaccine encoding the MOMP of *C. abortus*[51].

Later, other proteins have been proposed, such as those belonging to the family of Pmps, these have been shown to have the immunogenic potential for the development of immunogens against *C. abortus*[2]. One study evaluated Pmp18D in two different formulations, FL (tyrosine kinase 3 ligand type Fms; Flt3L) and *Vibrio cholerae* ghosts (VCGs), to induce innate and cross-protective immunity against genital *C. abortus* infection.

The evaluation was carried out with the regulation of the expression of the protein, by the activation and differentiation of different cell types. The results showed that the formulation that offers the best results is Pmp18D+VCG[52], as well as another variant, using an N-terminal fragment of this protein, called Pmp18D.1, the study evaluated its ability to induce an innate immune response in dendritic cells and activate the signaling pathways involved in the secretion of IL-1β[53]. Other proteins used as combined antigens (MIP and CPAF) demonstrated 50 % efficacy against a commercial live attenuated vaccine; additionally, although there is release of the pathogen by immunized animals, these are released in smaller quantities compared to the negative control. Nevertheless, it was possible to observe in this study that, when these two proteins are administered individually, it has no effect against the experimental infection[54].

Several studies have been carried out against *C. psittaci*, using different types of proteins. Initially focused on the evaluation of the persistence of a DNA vaccine (pcDNA1-MOMP) and the expression of the recombinant protein (rMOMP) from the MOMP of an avian strain of *C. psittaci*, which causes respiratory problems in turkeys, the results showed that the persistence of the vaccine was 10 weeks and the expression of the protein was proportional to the persistence time[55]. Subsequently, using a recombinant adenovirus vaccine using the same protein, it was evaluated in birds against avian chlamydia, the results of this study showed that this vaccine was safe, and that the protection rate reached up to 90 % in the challenged animals. Although the period of protection of the vaccine was six months, it is emphasized that the growth periods of the birds used for meat are approximately similar. However, birds intended for laying should be vaccinated twice because they have longer life spans[56].

On the other hand, Pmps have also been used as candidates for the development of vaccines against *C. psittaci*; in this sense, a study developed and examined a recombinant vaccine administered due to the herpesvirus of turkeys, using the 5' end of the PmpD gene, which encodes the N-terminal fraction of this (pmpD-N). The evaluation of the recombinant virus (rHVT-pmpD-N) in the challenged birds revealed increased levels of specific antibodies against PmpD and a proliferation of specific lymphocytes against it. After the challenge with the strain *C. psittaci* CB7, a significant decrease in respiratory distress, lesions and bacterial load was found in the challenged group[57]. One study evaluated the efficacy of vaccination with plasmid proteins to prevent lung infection by *C. psittaci* in mice, in which a recombinant protein of CPSIT_p8 is used, which belongs to an important virulence factor in the form of a highly conserved "cryptic" plasmid of 7.5 kb. A recombinant of this protein was produced and challenged in a murine model. The results in this study showed that immunization significantly decreased the bacterial load in the lungs of the challenged mice, a lower level of IFN-γ was also observed. Its results conclude that the recombinant protein evaluated in this study induces significant protective immunity against *C. psittaci* and that it could be

considered as a candidate for the development of a new vaccine for the prevention of infections caused by this bacterium[58].

However, other proteins involved in the virulence of this bacterium, such as chlamydial inclusion membrane proteins (Incs), have also been used as candidates in the development of vaccine prototypes. One study employed a recombinant of the transmembrane head protein CPSIT_0846 and challenged mice with respiratory tract infection caused by *C. psittaci*, the study revealed a strong cytokine profile with high levels of IFN-γ; similarly, a strong humoral immune response was detected in the challenged mice, with high titers of specific IgG antibodies. The strong immune response correlated with a significantly reduced bacterial concentration and a decrease in the inflammatory pathology in the lungs of the mice after the challenge. The results of this study suggest that the protein CPSIT_0846 may be a possible candidate antigen for the development of a vaccine to induce protection against this type of infections[59].

For the detection of antibodies against *C. psittaci*, an ELISA test based on the N-terminal fragment of the PmpD (PmpD-N) was developed, the tests were performed to determine its sensitivity and specificity in experimentally infected and uninfected birds. The results of this study revealed that the ELISA-PmpD-N had a sensitivity and specificity of 97.9 %, 100 % respectively; in addition, there was no cross-reaction with positive serums for other avian pathogens. The results concluded that this protein fraction (PmpD-N) can be used as an antigen for the diagnosis of *C. psittaci* infections in birds[60]. It should be noted that all studies have been carried out in *C. psittaci* of avian origin; however, since *C. psittaci* is genetically related to *C. abortus*[61], with this evidence, the idea of focusing studies on the variant that affects small ruminants can be contemplated.

In *C. pecorum*, the most recent study focused on the development of vaccine prototypes using surface proteins has been carried out in experimentally infected ewes and evaluating two recombinant proteins: rMOMP and rPmpG, this study identified B-cell epitopes in asymptomatic animals, with arthritis related to this agent and animals immunized with a recombinant vaccine of these proteins. The results of this study conclude that these tests can help improve diagnostic tests for this agent in sheep flocks[62].

Later, a study evaluates a direct ELISA test using two recombinant protein antigens of this bacterial species (rPmpG and rMOMP-G) and using the Pepscan method, a mapping and characterization of B-cell epitopes in these proteins was carried out in lambs with asymptomatic *C. pecorum* infections, with polyarthritis associated with *C. pecorum* and vaccinated with recombinant proteins. The results revealed that there is an immune response of antibodies against PmpG in the natural infection. Antibodies against MOMP-G increased in animals with polyarthritis. Finally, an epitope response was identified in immunized lambs and in naturally infected lambs[63].

Conclusions

Studies focused on the identification of immunoreactive proteins for the development of ELISA tests and vaccine prototypes against diseases caused by species of the genus *Chlamydia* that affect small ruminants have become very relevant in recent years, due to the importance in public health, animal welfare and economic importance that they represent.

Immunoassays with specific proteins of each species, such as Pmps, can be a key point to avoid cross-reactions between species, which would reduce erroneous results in veterinary diagnostic laboratories.

C. abortus is the species of the genus that has been the most importance in the last decade; since the available commercial vaccines have not given satisfactory results for the prevention and control of OEA; in addition to the biological risk that it represents. The use of subunit vaccines as an option for prototype development has good levels of safety compared to commercial vaccines; since they do not represent a risk to the personnel who handles them and offer results equal to or superior to those offered by them.

Conflicts of interest

The authors declare that they have no conflict of interest.

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