Efficacy of Massachusetts and 793B Vaccines Against Infectious Bronchitis Moroccan-Italy 02 Virus in Specific-Pathogen-Free Chickens and Commercial Broilers

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SUMMARY. The ability of commercial vaccines H120 and 4/91 to protect against Moroccan-Italy 02 infectious bronchitis virus (Mor-It02) was investigated in specific-pathogen-free (SPF) chickens and commercial broiler chickens. Commercial broiler chicks (Experiment 1) were vaccinated at the hatchery with H120 vaccine at Day 1, and challenged at Day 21 with 10^4 50% egg-infective dose (EID_{50}) of Mor-It02. All chicks were observed daily for clinical signs attributable to Mor-It02 infection during the 10 days postchallenge (pc). At 5 and 10 days pc, chicks were humanely sacrificed for necropsy examination, and tissues were collected for histopathology evaluation. To better understand the findings on commercial broilers, day-old SPF chicks were divided into five groups in a second experiment: Group Mass/4-91, vaccinated with H120 and 4/91 respectively at Days 1 and 15 of age; Group Mass/Mass, vaccinated by H120 at Days 1 and 15; Group Mass, vaccinated with H120 at Day 1; Group NV, kept unvaccinated; and Group NC, kept as a negative control (unchallenged). At Day 24 of age, Groups Mass/4-91, Mass/Mass, Mass, and NV were challenged with 10^4 EID_{50} of Mor-It02. In both experiments, blood samples were collected at different periods for serologic analyses. Oropharyngeal swabs were collected for virus detection by reverse-transcription PCR. In Experiments 1 and 2, respiratory signs started as early as 24 hr pc and maximum severity was observed on Days 3 and 4 pc. The viral shedding rate was significantly lower in Group Mass/4-91 compared to other challenged groups. Serologic analysis in both experiments showed that the sera of challenged group exhibited significantly higher antibody titers than sera collected before challenge. Histopathologic investigations in SPF birds showed deciliation and hyperplasia in Group NV and less-pronounced lesions in Groups Mass/Mass and Mass. In commercial broilers vaccinated with H120 alone, hyperplasia and deciliation were observed in 90% of the tracheas. These experiments illustrated that Mor-It02 is pathogenic for chickens and a combination of live H120 and 4/91 vaccines given respectively at Day 1 and Day 15 of age confer a good protection against Mor-It02.

RESUMEN. Eficacia de las vacunas Massachusetts y 793B contra el virus de la bronquitis infecciosa marroquí-Italia 02 en pollos libres de patógenos específicos y en pollos de engorde comerciales.

Se investigó la capacidad de las vacunas comerciales H120 y 4/91 para proteger contra el virus de la bronquitis infecciosa marroquí-Italia 02 (Mor-It02) en pollos libres de patógenos específicos (SPF) y en pollos de engorde comerciales. Se vacunaron pollos de engorde comerciales (Experimento 1) en la planta incubadora con la vacuna H120 al día 1 y se desafiaron al día 21 con 10^4 dosis infectantes para embrión de pollo 50% (EID_{50}) de la cepa Mor-It02. Todos los pollos se observaron diariamente para detectar signos clínicos atribuibles a la infección por el virus Mor-It02 durante los 10 días posteriores al desafío. A los 5 y 10 días después del desafío, se practicó la eutanasia de los pollos para realizar el examen de necropsia y los tejidos fueron recolectados para la evaluación histopatológica. Para comprender mejor los hallazgos en pollos de engorde comerciales, los pollos libres de patógenos específicos de un día se dividieron en cinco grupos en un segundo experimento: Grupo Mass/4-91, vacunados con H120 y 4/91 a los días 1 y 15, respectivamente; Grupo Mass/Mass, vacunados con H120 a los días 1 y 15; Grupo Mass, vacunados con H120 al primer día de edad; Grupo NV, que se mantuvo sin vacunar; Grupo NC, mantenido sin desafiar como un control negativo. Al día 24 de edad, los grupos Mass/4-91, Mass/Mass, Mass y NV fueron desafiados con 10^4 EID_{50} del virus Mor-It02. En ambos experimentos, se recolectaron muestras de sangre en diferentes periodos para análisis serológicos. Se recolectaron hisopos orofaríngeos para la detección del virus mediante transcripción reversa y PCR. En los experimentos 1 y 2, los signos respiratorios empezaron tan temprano como 24 h después del desafío y presentaron la severidad más alta en los días 3 y 4 después del desafío. La tasa de eliminación viral fue significativamente menor en el grupo Mass/4-91 en comparación con otros grupos desafeados. El análisis serológico en ambos experimentos mostró que los sueros del grupo desafiado exhibían títulos de anticuerpos significativamente más altos que los sueros recogidos antes del desafío. Las investigaciones histopatológicas en aves libres de patógenos específicos mostraron pérdida de cílios e hiperplasia en el grupo NV y lesiones menos pronunciadas en los grupos Mass/Mass y Mass. En pollos comerciales vacunados con H120 solo se observó hiperplasia y pérdida de cílios en el 90% de las tráqueas. Estos experimentos mostraron que el virus Mor-It02 es patógeno para pollos y una combinación de vacunas vivas H120 y 4/91 administradas respectivamente en el día 1 y día 15 de edad confieren una buena protección contra el virus Mor-It02.

Key words: Moroccan-Italy 02 virus, infectious bronchitis virus, Massachusetts, 793B vaccine, specific-pathogen-free chicken, commercial broiler

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Infectious bronchitis (IB) is a highly infectious disease of chickens and has worldwide distribution. IB virus (IBV) is extremely difficult to control because it has extensive genetic diversity and a high mutation rate. It continues to cause disease in chickens, even in vaccinated birds because the virus is constantly changing and evolving to avoid the bird’s immune response (20). Mutation and recombination processes have been shown to be involved in genetic variation and thus in the evolution of IBV, leading to the emergence of new variant strains (24). The molecular analyses carried out by Dolz et al. have shown that vaccination contributes greatly to the creation of a beneficial environment that leads to the successful diffusion of new genotypes (11).

IB is characterized by respiratory signs including gasping, coughing, sneezing, tracheal rales, and nasal discharge and it is known to be involved in poor egg production and poor egg quality in layers (14). Some strains of IBV could cause an interstitial nephritis (18). During the last decade, the Moroccan poultry industry has observed an increasing incidence of respiratory diseases related to IBV infections in vaccinated and unvaccinated flocks leading to severe economic losses (15). Several reports on IBV in Morocco pointed out its economical importance during the last three decades; IBV was found to be responsible for respiratory and renal diseases and drop in egg production associated with poor egg quality (1,13,14). Epidemiologic investigations carried out by Fellahi et al. (15) revealed that the Italy 02 IBV genotype had a prevalence of 32% between 2010 and 2014 while Massachusetts and 793B genotypes accounted for 66% and 2%, respectively. It was the first detection of this genotype in Morocco and Africa and the Moroccan-Italy 02 IBV (Mor-It02) was shown genetically different from the reference strains of Italy 02 (15). The deduced amino-acid sequences of Mor-It02 isolate proteins demonstrated that the Moroccan isolates represent a unique variant compared to the sequences of Mor-It02 isolate proteins demonstrated that the same virus, but the extent of protection against challenge with the Mor-It02 virus. It was already demonstrated by Jones et al. in 2005 that the combination of two heterologous vaccines (IBV Massachusetts type with a booster with Arkansas or D274) gave excellent protection against the challenge of Italy 02 (22). The objective of this study is to investigate the possibility whether the combination of H120 + 793B vaccines could protect birds against a challenge with the Mor-It02 virus.

Abbreviations: CE = chicken embryo; EID<sub>50</sub> = 50% egg infective dose; IB = infectious bronchitis; IBV = infectious bronchitis virus; Mor-It02 = Moroccan-Italy 02; Nt = not tested; pc = postchallenge; qRT-PCR = real-time reverse-transcription PCR; RT-PCR = reverse-transcription PCR; SPF = specific-pathogen-free

**MATERIALS AND METHODS**

**Birds.** Thirty-one Moroccan commercial broiler chicks had been vaccinated at 1 day old in the hatchery against IBV with a full dose of H120 and Newcastle disease virus as routinely applied using a sprayer cabinet. Birds were transferred to two separate pens at the Avian Pathology Unit at the Agronomy and Veterinary Institute (Rabat, Morocco).

Fifty-five specific-pathogen-free (SPF) chicks were obtained from the National Institute for Agronomy Research, Plate-Forme d’Infectiologie Expérimentale (Nouzilly, France) at 1 day old and divided into five groups in poultry isolators at the Joined Research Unit Host-Pathogen Interactions (1225) at the National Veterinary School of Toulouse (France).

Experimentations were conducted in accordance with European and French legislations on laboratory animal care and use (French Decree 2001–464 and European Directive CEE86/609) and animal protocols approved by the Ethics Committee “Sciences et santé animale,” committee number 115. The birds were kept in within the animal facilities (biosafety level 2) of the National Veterinary School of Toulouse (Experimental unit agreement number C31555277) and had access to food and water ad libitum.

**Vaccines.** IB commercial attenuated vaccines used in this study were MassH120 (Bioral H120, batch no. L418386, Merial, Lyon, France) and 4/91 (Nobilis IB 4-91, batch no. A188CJ01, 793B genotype, MSD, Lyon, France).

**Virus.** The challenge virus γCoV/chicken/Morocco/I38/2014, referred as Mor-It02 (accession no. KJ701020), was used. This virus had been isolated from an outbreak associated with mortality and respiratory disease in a broiler flock (16,17). Challenge virus titration was performed using 10-day-old SPF chicken embryos (CEs) inoculated via the allantoic cavity. The CEs were examined for IBV lesions (curling and dwarfing) up to 6 days postinoculation. Viral titers were calculated according to Reed and Muench (28) and expressed as the 50% egg-infective dose per milliliter (EID<sub>50</sub>/ml).

**Vaccine study.** *Experiment 1.* Commercial broiler chicks vaccinated at hatch by spray with full dose of H120 were divided into two groups. Group 1 birds (n = 21 birds) were tagged then challenged at Day 21 of age by oculo-nasal routes with 10<sup>4</sup> EID<sub>50</sub> of Mor-It02 strain in a 200μl volume. Group 2 birds (n = 10 birds) were kept as unchallenged control group.

*Experiment 2.* Fifty-five SPF chicks were divided into five groups in five separate isolators: Group Mass/4-91, vaccinated with H120 at Day 1 and boosted by 4/91 at Day 15; Group Mass/Mass, vaccinated twice with H120 at Days 1 and 15; Group Mass, vaccinated with H120 at Day 1 only; Group NV, not vaccinated; and Group NC, not vaccinated and not challenged. At 24 days of age, birds of groups Mass/4-91, Mass/Mass, Mass, and NV were individually tagged then challenged with 10<sup>4</sup> EID<sub>50</sub> of Mor-It02 strain via the oculo-nasal route in a 200-μl volume. Group NC was left as a negative control.

The SPF birds were vaccinated with commercially available Mass H120 (3.7–5.0 log<sub>10</sub> EID<sub>50</sub>) and IB 4/91 (3.6 log<sub>10</sub>EID<sub>50</sub>) vaccines delivered in 0.2ml/dose via the ocular and nasal routes as recommended by the vaccines’ manufacturers.

**Clinical observations.** After challenge, all birds were examined individually for respiratory signs and scored as follows: −, no respiratory sign; +, mild signs of respiratory distress without rales; ++, moderate signs of distress with rales; ++++, severe signs of distress with severe rales.
Table 1. Protection conferred by the H120 vaccine against the Mort-It 02 IBV in commericial broilers as shown by clinical signs, macroscopic and microscopic lesions, and seroconversion.

<table>
<thead>
<tr>
<th>Bird no.</th>
<th>Respiratory signs*</th>
<th>Gross lesions in trachea</th>
<th>Gross lesions in air sacs</th>
<th>Histologic lesions</th>
<th>Serologic results 10 days after challenge</th>
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*aOnly injected birds are recorded here. None of the birds in the control group showed respiratory signs or macroscopic or microscopic lesions, nor did they seroconvert.

*bBirddes were scored as follows: +, no clinical sign; ++, respiratory distress without rales; ++++, presence of rales; +++, severe rales.

*ND = Not done. Birds 1–11 were euthanatized at 5 days postchallenge; birds 12–21 were euthanatized at 10 days postchallenge.

Postmortem examinations. In commercial broilers, postmortem examination was performed on 11 birds from the challenged group and two birds from the unchallenged group. The remaining birds were sacrificed at Day 10.

In SPF chicks, at 5 days postchallenge (pc), six birds from each challenged group and two birds from the NC group were sacrificed and macroscopic lesions observed in all organs were recorded. The remaining birds were sacrificed at Day 12. All gross lesions were recorded.

Serologic analysis. In Experiment 1, blood samples were collected from 10 birds at Day 20 (1 day before challenge) and 10 days after challenge. A commercial ELISA kit (IDEXX, Kansas City, MO) was used to assess the antibody response.

In Experiment 2, blood samples were collected at Days 15, 23 and 36 of age, in order to determine the antibody response after vaccination and following challenge. The level of antibody to IBV was measured by the IDEXX ELISA kit (Hoofddorp, The Netherlands) and expressed according to the manufacturer’s instructions.

Real-time reverse-transcription PCR (qRT-PCR). Virus detection was performed on oropharyngeal swabs collected from each bird on Days 3, 5, and 7 and tested individually. Viral load was determined by qRT-PCR performed on RNA extracted from swabs. Viral RNA was extracted using the Macherey Nagel viral RNA extraction kit (Düren, Germany) following the instructions of the manufacturer. The extracted RNA was subjected to qRT-PCR, which was performed in two steps. First, a reverse transcription was carried out using the RevertAid kit (Thermo Fisher, Waltham, MA) with two mixes: Mix 1 (6.5 μl nanopure water, 1 μl random hexamer, 5 μl RNA) was incubated at 65 C for 5 min. Mix 2 (4 μl 5X buffer, 2 μl dNTPs (10 μM), 0.5 μl RNAse Out, 1 μl Revert Aid) was then incubated at 25 C for 10 min, 42 C for 60 min, and 70 C for 10 min. The reaction was carried out in an Applied Biosystems thermocycler (Foster City, CA).

qRT-PCR for IBV nucleoprotein gene detection was performed using the primers and probe described by Meir et al. (26). PCR was carried out using a Thermo Fisher scientific kit. The reaction volume contained 5 μl of 2X RT-PCR buffer mix, 2.5 μl nucleic-acid-free water, 0.2 μl AIBV-forward primer (10 μM), 0.2 μl AIBV-reverse primer (10 μM), 0.1 μl AIBV-TM probe, 2 μl complementary DNA with the following program: initial denaturation at 94 C for 2 min, followed by 40 cycles of denaturation at 94 C for 10 sec and hybridization/elongation for 30 sec at 60 C. A Light Cycler 480 (Roche, Penzberg, Germany) was used.

Histopathologic lesions. At 5 days pc, tracheas from euthanatized birds were collected and fixed in 10% formalin. After fixation, tissues were processed in paraffin blocks, sectioned at 4 μm, and stained with hematoxylin and eosin for microscopic examination.

RESULTS

Clinical observations. Experiment 1. The respiratory signs started as early as 24 hr pc in 2 birds out of 21. At Day 2 pc, symptoms were observed in 75% of the birds. The respiratory signs varied from mild respiratory distress to severe rales. The maximum severity was observed on Day 4 pc in 81% of the birds. These clinical signs continued up to Day 5 pc in 76% of the birds, then the signs started to decrease progressively and at Day 10 pc only 30% of the birds showed mild respiratory distress. In the control group, no clinical signs were observed (Table 1).

Experiment 2. Daily clinical observations of SPF chickens revealed the presence of respiratory signs at 1 day pc in all groups, with mild respiratory distress in Group Mass/Mass and severe signs in Groups Mass/Mass, Mass, and NV. Birds in Group Mass/Mass showed a peak of respiratory signs in 72% of the birds at Day 2 pc. These signs regressed progressively. In Group Mass/Mass, maximum of severity was observed between Days 2 and 4 pc in 81% of the birds and the signs persisted in 80% of the birds until Day 10 pc. In Group Mass, all birds showed respiratory signs during the 10 days pc. In Group NV the respiratory signs were more severe at Days 7 and 10. However, no clinical signs were observed in birds of the Group NC (unchallenged; Table 2).

Postmortem examination. Experiment 1. The macroscopic lesions on Day 5 pc in birds vaccinated with H120 and challenged were as follows: congestion and presence of petechiae in the trachea, pharynx, and larynx in 90% of the birds. Others lesions, such as airsacculitis and air sacs thickening were observed in 45% of the birds. Lesions of pericarditis were found in 18% of the birds.

At Day 10 pc the macroscopic examination revealed similar lesions, with 40% of the birds showing lesions in the upper respiratory tract, and 50% of the birds with lesions in the air sacs. Moreover, one bird presented a cyst at the right oviduct. The unchallenged control birds did not show any pathologic lesions (Table 1).

Experiment 2. The postmortem examination carried out on Day 5 pc revealed congestion and presence of petechiae in the trachea, pharynx, and larynx in 50% of the birds in Group Mass/4-91, 83% in Group Mass/Mass, 50% in Group Mass, and 100% in Group NV. Other lesions were observed such as airsacculitis and thickening of air sacs in 83% of the birds of Group NV. Less pronounced lesions were found in 50% of the birds of Group Mass. No gross lesions were detected in the unchallenged group.

At 12 days pc, 40% of the birds in Group Mass presented petechiae in the trachea, pharynx, and larynx. In Group NV, in
addition to the lesions described in Group Mass, air sac lesions were observed in 100% of the birds (Table 2).

**Serologic analysis.** While commercial birds were all seronegative before IBV challenge despite having been vaccinated in the hatchery, vaccinated SPF birds all seroconverted before challenge (no antibodies detected in the NV or NC groups). Higher ELISA titers were detected in the Mass/4-91 and Mass/Mass groups than in the Mass group. As expected, birds showed increased ELISA antibody titers after challenge in both experiments (Tables 1, 2).

**Virus shedding in SPF birds.** The virus detection by qRT-PCR from oropharyngeal swabs in Group Mass/4-91 revealed that 27% of the samples were positive on Day 3, 54% were positive on Day 5, and no birds tested positive on Day 7 pc. In Group Mass/Mass, 54% of the swabs were positive on Days 3 and 5, and 40% of the birds were positives on Day 7 pc. In Group Mass, 45% of the birds were positive on Days 3 and 5, and 60% were positive on Day 7. In contrast, in Group NV, 100% of the birds were positives on Days 3, 5, and 7 pc (Table 2).

**Histopathologic lesions.** *Experiment 1*. The histopathologic examinations of tracheas collected from challenged birds on Day 5 revealed that 90% of the tracheas showed hyperplasia and deciliation at the epithelium surface. In the lamina propria, hyperemia and diffuse or localized infiltration by inflammatory cells and follicles were observed. Hyperemia was observed discreetly in 100% of the samples. However, in 50% of the tracheas diffuse infiltration by inflammatory cells was found. Follicles in the form of localized infiltrates were found in 30% of the samples (Table 1).

*Experiment 2*. The histopathologic investigations on Day 5 pc did not show any significant changes in tracheas in Group Mass/4-91; however, in Group NV, five birds out of six showed deciliation and hyperplasia of the epithelium surface. Likewise, less pronounced lesions in comparison to Group NV were observed in Groups Mass/ Mass and Mass. In all groups, diffuse lymphocytic infiltrations of the lamina propria of the laryngeal and tracheal respiratory mucosa were found (Table 2).

**DISCUSSION**

IB is one of the most economically significant diseases of the poultry industry in the world. Vaccination programs rely mainly on the use of the Massachusetts strain, which is the most commonly used IBV vaccine strain in Morocco; however, presence of the disease in vaccinated chickens is commonly observed (1,12,15). A new Moroccan variant of the Italy 02 genotype was isolated for the first time in Morocco and in Africa between 2010 and 2014 as the second most frequently detected genotype (prevalence: 32%) (15). The results of clinical protection in field conditions revealed that the peak of disease severity was observed on Day 4 in 81% of the birds, and the intensity of clinical signs suggested an inefficient vaccination. Gelb et al. previously reported similar findings (17). In SPF chicks, the immunization with H120 at Day 1 followed by 4/91 at Day 15 (Group Mass/4-91) provided chickens with full protection from overt clinical disease caused by the challenge Mor-It02 virulent strain after 3 days pc. In contrast, in Groups Mass/Mass and Mass symptoms persisted beyond Day 7 pc. The results of this study confirm previously reported studies that combination of two different vaccines gives better protection than one vaccination against challenge with an unrelated IBV (7,22). In addition, this study illustrates that under field and experimental conditions, IBV vaccinations significantly reduced disease outcome, indicating that clinical protection could be achieved by selecting appropriate vaccines. This result corroborates several previously published studies (3,29,31).

In the Mor-It02–infected SPF chicks, necropsy examination at 5 days pc revealed congestion and presence of petechiae in the trachea, pharynx, and larynx in 50% of the birds in Groups Mass/4-91 and Mass, 83% in Group Mass/Mass, and 100% in the unvaccinated Group NV. These lesions persisted in 40% of the birds in Group Mass and up to 12 days pc in 100% of birds of Group NV. These
results indicate that the lesions disappeared in the groups that received a booster vaccination at 14 days. Previous studies demonstrated that a Massachusetts and 4/91 IB vaccination protocol does not affect protection against homologous IBV strains (Ma5 or 4/91) while inducing high protection against heterologous IBV types as we found in this study against Mor-It02 virus (6, 7).

In Group Mass/4-91, at 7 days pc, virus was detected in none of the birds; however, in Groups Mass/Mass and Mass virus was still present. In Groups Mass/Mass and Mass, ongoing viral replication even after 7 days probably stimulated the production of antibodies. In Group NV we observed seronegative birds 12 days pc, which might be explained by the fact that antibody production takes longer at the first contact with the antigen. Recently, Smialek et al. demonstrated that the birds vaccinated with Ma5 and 4/91 strains developed a stronger immune response than their counterparts that received Mass or 4/91 alone as shown by levels of CD8+ T cells stimulation in spleen and Harderian gland, and levels of IgA and IgY in upper respiratory tract washings and serum (30).

The serologic results obtained in commercial birds indicate that all birds previously vaccinated at the hatchery by spray with the H120 vaccine were seronegative against IB before challenge. This might be related to a vaccination failure, or maternal antibodies could have interfered with vaccination, while not lasting long enough to still be present upon challenge. However, previous reports have indicated that maternal antibodies to IBV do not interfere with IBV vaccination (8, 9, 10). The authors reported that the presence of high levels of circulating maternal antibodies did not prevent effective immunization against IBV when the chicks were vaccinated at 1 day of age, presumably because the antibodies circulating in the blood do not interfere with the local immune response to vaccination, particularly in the Harderian gland (9). After infection, serologic tests showed heterogeneity in the bird’s seroconversion. Cavanagh showed that, in general, 10% of vaccinated chickens show no protective response against infection by a homologous virulent strain, which may explain serologic results after infection in commercial birds (4).

The virus detection by qRT-PCR in Group Mass/4-91 revealed that samples were positive on Days 3 and 5; however, no birds tested positive on Day 7. In contrast, the viral shedding rate was positive in Groups Mass/Mass, Mass, and NV on Days 3, 5, and 7. The proportion of positive samples in the Group Mass/4-91 was significantly lower than that of the others groups. In other words, we observed that birds receiving two vaccinations (H120 at 1 day and 4/91 at 15 days of age) cleared the virus earlier than birds that received a single H120 vaccine at 1 day of age, so one would expect a lower transmission of the virus. Jackwood et al. reported that a reduced challenge virus shedding in vaccinated birds is important and should be considered when evaluating protection (21). The microscopic findings in the SPF and broiler chicks following Mor-It02 infection were similar to other virulent IBVs (2, 5, 27). In both types of birds, severe and early onset of histopathologic lesions was seen in the trachea. Additionally, fewer lesions were observed in Group Mass/4-91 compared to other groups, a finding similar to the results obtained for other field strains in several previous studies (6, 7, 32).

Conclusions. The results obtained in this study showed that the use of H120 vaccine at 1 day of age and 4/91 at 15 days of age confers good protection of chickens against Mor-It02; therefore this program could be recommended to farms in Morocco, as was previously recommended in several studies. The surveillance of IBV serotypes/genotypes should be continued to understand virus circulation and adapt prophylactic programs to the field needs.

REFERENCES


Protection against Moroccan-Italy 02 infectious bronchitis virus


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