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Viral Agents Associated with Poult Enteritis and Mortality Syndrome: The Role of a Small Round Virus and a Turkey Coronavirus

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SUMMARY. Intestinal samples from turkey poults affected with poult enteritis and mortality syndrome (PEMS) were examined for viruses by immune electron microscopy and double-stranded RNA virus genome electropherotyping. Turkey coronavirus (TCV), avian rotaviruses, reovirus, and a yet undefined small round virus (SRV) were detected. The SRV and TCV were isolated and propagated in turkey embryos. Challenge of specific-pathogen-free turkey poults with SRV, TCV, or both resulted in mortality and clinical responses similar to those of natural PEMS. Our experiments indicate that SRV and TCV are possibly important agents in the etiology of PEMS and the combination of these infections might result in outbreaks with high mortality. The severity of clinical signs and mortality of PEMS are postulated to be partly related to the virus agents involved in individual outbreaks.

RESUMEN. Agentes virales asociados con el síndrome de mortalidad y enteritis en pavos: Papel de un virus pequeño y redondo y un coronavirus de pavo.

Se examinaron muestras intestinales de pavitos afectados con el síndrome de mortalidad y enteritis de pavitos para la presencia de virus mediante el microscopio electrónico y mediante la electroferotipificación. Se detectaron coronavirus de pavos, rotavirus aviares, reovirus y un virus pequeño redondo todavía no definido. El virus pequeño redondo y el coronavirus de pavo fueron aislados y propagados en embriones de pavo. El desafío de pavos libres de patógenos específicos con el virus pequeño y redondo, con el coronavirus del pavo ó con los dos virus, resultó en mortalidad y repuestas clínicas similares a las del síndrome de mortalidad y enteritis del pavo. Los experimentos indican que el virus pequeño y redondo y el coronavirus del pavo son agentes importantes en la etiología del síndrome de mortalidad y enteritis de pavitos y que la combinación de estas infecciones puede resultar en un brote de la enfermedad con alta mortalidad. Se cree que la severidad de los signos clínicos y la mortalidad del síndrome de mortalidad y enteritis del pavo están parcialmente relacionados con los agentes virales involucrados en brotes individuales.

Key words: poult enteritis and mortality syndrome, small round virus, turkey coronavirus

Abbreviations: DPI = days postinoculation; EID_{50} = mean embryo infective dose; GI = gastrointestinal; IEM = immune electron microscopy; PEMS = poult enteritis and mortality syndrome; PTA = phosphotungstic acid; SPF = specific-pathogen free; SRV = small round virus; TCV = turkey coronavirus

Poult enteritis and mortality syndrome (PEMS) is a transmissible disease commonly affecting young turkeys between 1 and 4 wk of age. The disease is characterized by diarrhea, anorexia, growth depression, immune dysfunction, and high mortality (1). PEMS has caused significant losses to turkey producers in North Carolina and several other southeastern states

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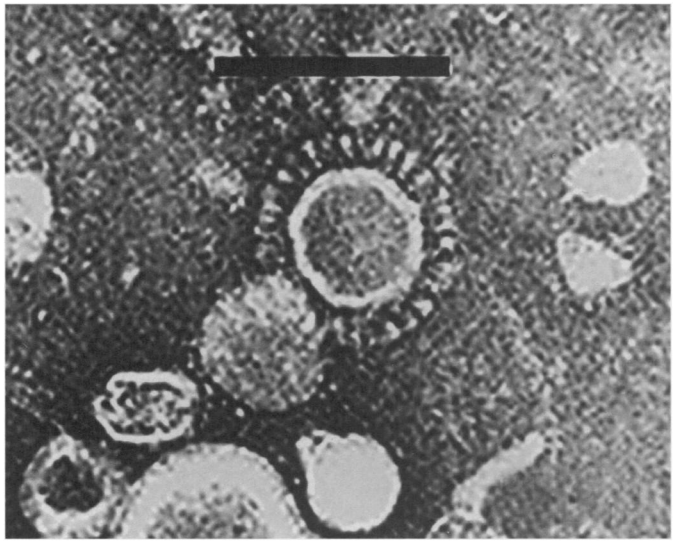

Fig. 1. Turkey coronavirus detected by immune electron microscopy in intestinal samples from poults affected by the poult enteritis and mortality syndrome. Bar = 100 nm.

since its recognition in 1991 (2). Although extensive research has been done, the etiology of the disease remains controversial. Coronavirus (6) and some other unidentified virus particles (4) as well as bacteria like *Escherichia coli* (3) have been implicated in the disease, but no etiologic relationship has been definitely established. In the present study, we detected several viruses in intestinal samples from PEMS-affected poults, including a turkey coronavirus (TCV) and a small round virus (SRV). The pathogenicities of the TCV and SRV were studied in experimental infection trials to elucidate their roles in the etiology of PEMS.

MATERIALS AND METHODS

Intestinal samples. Thirty-six samples of gastrointestinal (GI) tracts from 1-to-4-wk-old poults affected by PEMS were submitted to our laboratory over a 1-yr period. The samples were collected from 36 turkey flocks in 22 turkey farms in the states of North Carolina and Indiana. Samples were received frozen and contained one to six whole GI tracts.

Antisera. Convalescent sera were collected from poults that recovered from PEMS at about 2 wk after the onset of the disease and were submitted together with intestinal samples from the same flocks. The sera were inactivated at 56 C for 30 min and were then stored at −20 C until used.

Poults and embryos. All specific-pathogen-free (SPF) turkey embryos and SPF poults originated from the SPF flock maintained by the Food Animal Health Research Program. The flock is free of all recognized turkey pathogens, including all enteric viruses.

Immune electron microscopy (IEM). The GI tracts were thawed and the contents were stripped and homogenized 1/10 (w/v) in 0.05 M Tris-HCl buffer, pH 7.5, and clarified by centrifugation at 3000 × *g* for 30 min at 4 C. The supernatants were then filtered through 0.45-μm disposable syringe filters (Corning Glass Work, Corning, NY) and stored at −70 C until tested.

Two hundred microliters of the above supernatants were incubated overnight at 4 C with 200 μl of convalescent sera of the same flocks diluted 1/20 in 0.1 M sterile phosphate-buffered saline, pH 7.4. The dilutions for the supernatants and convalescent sera were chosen on the basis of preliminary trials. After incubation, the mixtures were ultracentrifuged for 15 min at 160,000 × *g* through a 50-μl cushion of 30% sucrose with a Beckman tabletop airfuge®. Pellets were resuspended in 400 μl of sterile distilled water and ultracentrifuged again as previously described (without sucrose cushion). The pellets were finally resuspended in 25 μl of sterile distilled water. One drop of the resuspended solution was placed on a carbon-coated 300 mesh Formvar® copper grid and stained with a drop of phosphotungstic acid (PTA) solution (3% PTA, 0.4% sucrose, pH 7.0). The grids were examined for viruses at 80 kV with a transmission electron microscope (Philips 201; Philips Norelco, Eindhoven, The Netherlands).

Virus isolation and propagation. Samples containing coronavirus only as identified by IEM were

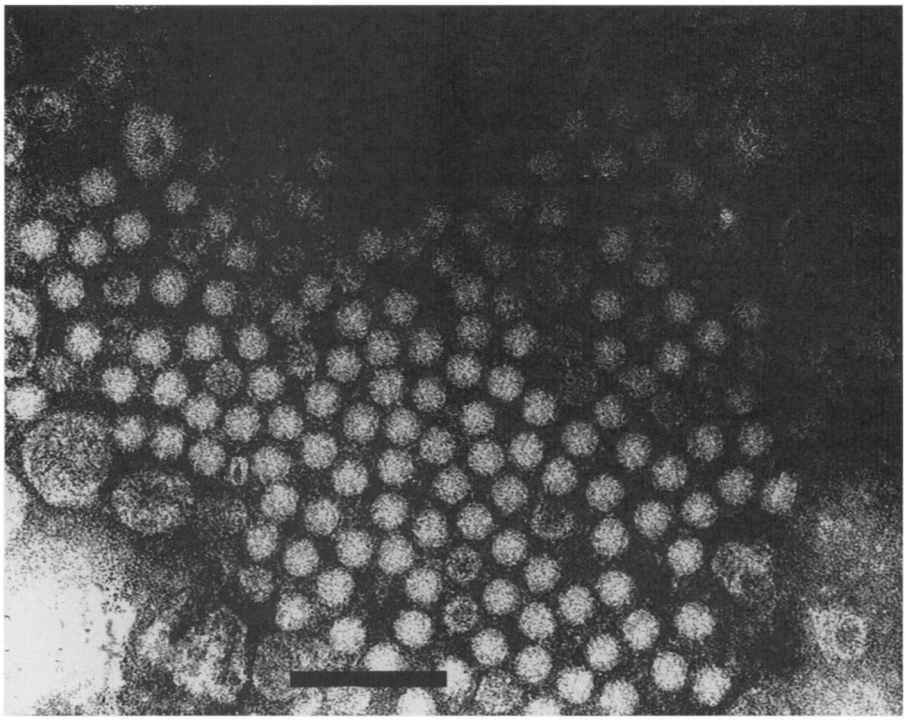

Fig. 2. The small round virus detected by immune electron microscopy in intestinal samples from poults affected by the poult enteritis and mortality syndrome. Bar = 100 nm.

used for virus propagation. About 0.2 ml of the GI tract content supernatant was inoculated into 22-day-old turkey embryos via the amniotic sac. After 3–4 days of incubation at 37 C, the intestines of the embryos were harvested, homogenized, and diluted 1/10 (w/v) in 0.05 M Tris-HCl buffer, pH 7.5. The homogenates were clarified by centrifugation at 3000 × *g* for 30 min at 4 C. The supernatants were then examined by IEM as described above and used for subsequent passages in turkey embryos.

For the SRV isolation and propagation, about 5 ml of the supernatant containing the SRV only as identified by IEM (filtered through 0.45-μm filter membrane earlier) was further filtered through 0.22-μm, 0.05-μm Millipore filter membranes. The final filtrate was used for experimental infection or was inoculated into 22-day-old SPF turkey embryos via the amniotic sac. Intestines of turkey embryos were collected and processed by following the same steps as for TCV propagation.

Both SRV and TCV were titrated in turkey embryos by a modification of a procedure described previously (22). Turkey embryo intestinal homogenates containing SRV or TCV were diluted serially (10^{-1}–10^{-7}) in 0.05 M Tris-HCl buffer, pH 7.5. Each dilution was inoculated to six SPF turkey embryos via the amniotic sac (0.2 ml/each). Embryos were considered infected when the intestines were enlarged at 3–4 days postinoculation (DPI). The mean embryo infective dose (EID_{50}) was estimated by the method of Reed and Muench (13).

Virus RNA extraction and electropherotyping. Double-stranded viral RNA extraction and polyacrylamide gel electrophoresis were done according to the procedure described by Theil *et al.* (20, 21). In brief, double-stranded RNA was extracted from 1–2 g of intestinal contents and was then subjected to electrophoresis in 7.5% polyacrylamide gel slabs with a vertical gel slab (Hoefer SE 600; Pharmacia Biotech, Piscataway, NJ). The gels were stained with silver nitrate and photographed.

Experimental infections. All SPF poults used in the different experimental groups were raised in wire cages inside high-security isolation rooms provided with HEPA-filtered intake and exhaust air. All the poults were provided with the same feed and water *ad libitum*. Different experimental groups were placed in separate rooms.

Trial 1. Twenty-one 7-day-old SPF turkey poults were inoculated orally with 0.1 ml each of the 0.05-μm filtrate described above. The titer of the SRV in the filtrate was not determined. The poults were wing-banded and placed together with another 14 poults that served as contact-exposed poults. Another 21 poults were not inoculated and served as unexposed controls. Poults were observed daily for clinical

signs. All poults were removed from the cages and weighed individually at 3, 4, 5, 7, 11, and 21 DPI. Two to six poults from each treatment were euthanatized at 3, 4, 5, 7, 11, and 21 DPI and examined for pathologic lesions. Intestinal samples were collected and examined for viruses by IEM.

Trial 2. Eighty-three 5-day-old SPF turkey poults were randomly separated into two groups. One group consisted of 56 poults, 27 of which were orally inoculated with 0.2 ml each of turkey embryo intestinal homogenate containing 10^3 EID_{50} SRV. The inoculated poults were wing-banded and placed together with the remaining 29 poults that served as contact-exposed poults. The other group of 27 poults was kept as noninoculated control. Poults were observed daily for clinical signs. Droppings were collected at 3, 4, 5, 6, and 7 DPI and used for IEM examination. Three to five poults from each group were necropsied at 3, 4, 5, 7, 14, and 21 DPI for examinations. The poults necropsied at 7, 14, and 21 DPI were weighed before euthanasia.

Trial 3. Thirty-six SPF poults were separated into four groups. Four 11-day-old poults in group I were inoculated orally with 0.2 ml each of turkey embryo intestinal homogenate containing 10^3 EID_{50} SRV. Four 11-day-old poults in group II were inoculated orally with 0.2 ml each of turkey embryo intestinal homogenate containing 10^2 EID_{50} TCV. Four 8-day-old poults in group III were inoculated orally with 0.2 ml each of turkey embryo intestinal homogenate containing 10^3 EID_{50} SRV, and the same poults were given 0.2 ml turkey embryo intestinal homogenate containing 10^2 EID_{50} TCV at 11 days of age. The remaining poults in each of the above groups were not inoculated and served as contact-exposed poults. Poults in group IV were not inoculated and served as a control group. The poults were observed daily for clinical signs. Three poults (one inoculated and two contacts) from each group were weighed and then euthanatized and examined for lesions at 2 and 4 DPI. All remaining poults were weighed and euthanatized at 7 DPI. Intestines were collected for IEM examinations.

Statistical analysis. Statistical comparisons of body weights between control poults and challenged poults were performed with the two-sample *t*-test. The *P*-value was for two-tailed *t*-tests.

RESULTS

Virus detection. The TCV (Fig. 1), SRV (Fig. 2), rotavirus, and reovirus were detected either alone or incombination with other viruses (Table 1). Rotaviruses were the most frequently detected viruses, followed by the SRV. Coronavirus was detected in 8 of 36 samples, and reovirus was detected in 5 of 36 samples.

Table 1. Viruses detected by immune electron microscopy, double-stranded RNA genome electropherotyping, or both in the gastrointestinal tracts of poults affected by the poult enteritis and mortality syndrome.

Virus	No. positive/ no. sampled examined[A]	Positive percentage
SRV	16/36	44%
TCV	8/36	22%
Rotaviruses[B]	26/36	72%
Reovirus[B]	5/36	14%
SRV + rotaviruses	8/36	25%
SRV + TCV	3/36	8%
SRV + TCV + rotaviruses	2/36	6%

[A]Samples originated from 36 flocks on 22 turkey farms and each sample consisted of contents from one to six gastrointestinal tracts.

[B]Rotaviruses were detected by either IEM or electropherotyping or both. Reovirus was detected by electropherotyping.

The SRV and rotaviruses in combination were detected in 8 of 36 samples. The SRV and TCV in combination were detected in 3 of 36 samples. A combination of TCV, SRV, and rotaviruses was detected in 2 of 36 samples. Avian rotavirus serogroups A, D, and F (17) and reovirus were detected by electropherotyping. Group D rotavirus was detected more frequently than the other serogroups (data not shown).

Virus isolation and propagation. The TCV was isolated and passaged serially in turkey embryos via the amniotic cavity. The TCV had all the typical morphologic features of coronaviruses. Embryos inoculated with TCV showed distinct intestinal lesions. The whole GI tract was distended and contained greenish contents. The embryos were usually stunted.

The SRV replicated in turkey embryos inoculated via the amniotic sac. The SRV was 30–32 nm in diameter and had no distinguishing surface features. Turkey embryos inoculated with SRV had distended intestines, and the gizzards were usually enlarged. The titer of SRV in the embryonic intestinal homogenate reached as high as 10^7 EID_{50}/ml.

Experimental infections. *Trial 1.* Inoculated and contact-exposed poults showed severe diarrhea with frothy watery droppings starting at 3 DPI and lasting for about 5 days. The morbidity was 100% and there was no mortality. The ceca were severely dilated and filled

Table 2. Mean body weights of 7-day-old SPF poults in trial 1 challenged with the small round virus originated from field outbreaks.

Treatment	Mean body weight (g) ± SD					
	3 DPI	4 DPI	5 DPI	7 DPI	11 DPI	21 DPI
Control	126.0 ± 4.6	147.6 ± 10.5	165.1 ± 10.3	187.7 ± 12.8	285.7 ± 30.1	530.4 ± 30.4
Inoculated	119.8 ± 4.6	131.1 ± 12.3*	133.9 ± 9.8*	156.5 ± 13.7*	232.6 ± 20.4*	407.7 ± 29.8*
Contact	130.6 ± 15.4	136.4 ± 6.6	139.8 ± 6.7*	150.9 ± 18.2*	209.6 ± 24.1*	425.7 ± 46.7*

* = significantly different from controls ($P < 0.05$).

with yellow-to-brown frothy contents. The poults were severely stunted and depressed, beginning as early as 4 DPI in inoculated poults and 5 DPI in contact-exposed poults. The body weights of these poults remained significantly depressed compared with the controls throughout the 21-day experimental period (Table 2). Some poults had pinpoint hemorrhages in the thymus that were not seen in the control poults. The SRV was detected by IEM in intestinal contents of challenged poults at 3, 4, 5, and 7 DPI, and no SRV was detected after 11 DPI.

Trial 2. Inoculated and contact-exposed poults had severe watery foamy diarrhea, anorexia, and depression starting at 3 DPI and lasting up to 7 DPI. Morbidity was 100%, whereas mortality was 5.6% (3/54; one inoculated poult died at 6 DPI and two contact-exposed poults died at 4 DPI).

The ceca were severely dilated with yellow foamy fluids. Other parts of the intestines were also filled with watery foamy contents. Some spleens were enlarged. Some poults had pinpoint hemorrhages in the thymus. The inoculated and contact-exposed poults showed significantly reduced weight gain at 7 DPI compared with control poults, but their body weight was similar to that of control poults by 14 and 21 DPI (Table 3). The SRV was detected by IEM in droppings between 3 and 7 DPI but not by 11 DPI.

Trial 3. The poults in the SRV-challenged group had symptoms and lesions similar to those of the poults trial 2. The poults in the TCV-challenged group had severe enteritis with yellowish loose droppings and were more depressed and stunted than poults in the SRV-challenged group. The intestines from the TCV-exposed poults were flaccid, thin-walled, and filled with loose contents, and the disease was acute, with symptoms appearing as early as 2 DPI and lasting for about 4 days. Poults in the group challenged with SRV plus TCV had more severe symptoms than poults in the groups challenged with SRV or TCV alone. The thymus of some poults in all the challenged groups had pinpoint hemorrhages. The mortality was as follows: SRV group, 11%; TCV group, 11%; SRV plus TCV group, 22%. The body weights of poults in all the challenged groups were significantly depressed by 7 DPI, whereas the TCV-alone and SRV plus TCV-challenged groups showed growth depression as early as 4 DPI (Table 4). The mean weight gains from 2 to 7 DPI in all the three challenged groups were much lower than those in the control group. The TCV plus SRV-challenged group was the lowest, followed by the TCV-only group and the SRV-only group. The TCV was detected by IEM at 2 and 4 DPI.

DISCUSSION

We have detected and isolated TCV and SRV from field samples of PEMS-affected poults. We have also demonstrated that both TCV and SRV were pathogenic and contagious and they initiated diseases similar to PEMS in SPF poults.

Poults challenged with SRV showed severe diarrhea, growth depression, and varied mortality in trials 1, 2, and 3. The mortality rates in trials 1, 2, and 3 were 0, 5.6%, and 11%, respectively. The growth depression in trials 1, 2, and 3 was inconsistent. In trial 1, significant growth depression occurred from 4 DPI through 21 DPI. In trial 2, the growth depression was significant only at 7 DPI, and the poults had body weight comparable to controls at 14 and 21 DPI. In trial 3, the growth depression was significant in all experimental groups at 7 DPI. The variations might be because of the difference in virus origin and dos-

Table 3. Mean body weights of 7-day-old SPF poults from trial 2 challenged with turkey embryo-propagated small round virus.

Treatment	Mean body weights (g) ± SD		
	7 DPI	14 DPI	21 DPI
Control	164.8 ± 36.0	263.0 ± 22.1	422.8 ± 27.9
Contact	119.2 ± 27.1*	272.8 ± 24.2	443.0 ± 18.9
Inoculated	142.3 ± 10.7*	282.4 ± 32.7	483.5 ± 45.1

* = significantly different from controls ($P < 0.05$).

age. The SRV induced a disease similar to the mild form of PEMS in turkey poults. Conceivably, under field conditions, the SRV infection in a flock of poults could cause significant consequences.

The TCV alone was able to cause severe enteritis, significant growth depression, and mortality in turkey poults. Although the prevalence of TCV as detected by IEM in this study was low, this might be because of the low sensitivity of IEM. Moreover, because the disease caused by TCV was very acute, the TCV could be detected by IEM only during a limited period after infection.

Poults challenged with SRV plus TCV showed the most severe clinical responses and the mortality was the highest. Because the mortality in PEMS is usually high, it is highly possible that most PEMS outbreaks are caused by concomitant or sequential infections of two or more viruses such as SRV and TCV.

SRV and TCV may be important agents in the etiology of PEMS. Barnes and Guy (1) speculated that TCV or some other viruses may be primarily responsible for initiating the enteritis, growth depression, and increased susceptibility to bacterial infections, which might account for the mortality. Our experiments indicate that SRV and TCV not only initiate enteritis and growth depression but also cause mortality. We conclude that a combined infection of SRV and TCV could be responsible for the outbreaks of the severe forms of PEMS, whereas an infection by SRV or TCV alone can initiate different milder forms of PEMS.

Rotaviruses, especially serogroup D, were the most frequently detected viruses. This finding was consistent with a previous report (18). In experimentally infected turkeys, turkey rotaviruses did not cause mortality. Under field conditions, clinical signs caused by rotavirus infection varied in severity, with diarrhea and wet litter as the predominant signs (9). In our study, rotaviruses were frequently found in combination with other viruses, such as SRV and TCV. Rotaviruses had been detected in combination with other viruses in diarrheic turkey poults in earlier studies (15,18). Their pathogenicity in combination with SRV, TCV, or both in the etiology of PEMS needs to be evaluated.

Reovirus was the least detected virus in this study. Reovirus has been found in feces of healthy turkey poults in other studies in our laboratory (unpubl. data). Because reovirus can be commonly found in the digestive and respiratory tracts of clinically normal chickens and turkeys (16), it is unlikely to play an important role in the etiology of PEMS.

Table 4. Mean body weights, mean body weight gains, and mortality in SPF poults from trial 3 challenged with the SRV, the TCV, and the SRV plus the TCV.

Treatment	Mean body weights (g) ± SD			Mean body weight gain (g) from 2 to 7 DPI	Mortality
	2 DPI	4 DPI	7 DPI		
Control	143.3 ± 33.1	188.7 ± 27.3	283.3 ± 9.61	140.0	0% (0/9)
TCV	97.3 ± 33.1	108.7 ± 36.2*	161.5 ± 19.1*	64.2	11% (1/9)
SRV	146.3 ± 21.9	194.0 ± 52.3	253.2 ± 21.8*	106.9	11% (1/9)
SRV + TCV	117.7 ± 41.0	137.3 ± 14.0*	136.3 ± 38.0*	18.6	22% (2/9)

* = significantly different from controls ($P < 0.05$).

The SRV or TCV infection caused hemorrhagic lesions in the thymus, which raised the question of a possible effect on the turkey immune system. In another study (12), SRV and TCV were thought to predispose the poults to infection by other "opportunistic" agents, such as *E. coli.* This may happen via either a permanent or transitory blockage or dysfunction of the immune system. The PEMS poults have been shown to exhibit immune dysfunction at both cellular and humoral levels (8,10,11). SRV seems to be an agent that can potentially affect the immune system.

The small round structured viruses include calicivirus, astrovirus, and enterovirus. Few studies have been reported about astrovirus (14) and enteroviruslike viruses associated with poult diarrhea (5,7,19). The SRV described in this report does not have the distinguishing surface features of the astroviruses, though the size is similar. The enteroviruslike virus was reported to be 18–24 nm in size, much smaller than the SRV. The size of known caliciviruses is between 30 and 40 nm, and some strains have distinguishing surface features such as circular oval surface hollow and scalloped feathery outer edge. Characterization of the SRV is in progress in our laboratory.

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