FIRST RECORD OF *HETERODERA FILIPJEVI* IN NORWAY

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Summary. During the years 1995 to1999 a survey was carried out to study the occurrence of *Heterodera* spp. in cereals in Norway. Cereal cyst nematodes were found widespread in all the principal cereal growing areas. A formerly unidentified species of cereal cyst nematode, belonging to the "*Heterodera avenae* complex", is recorded for the first time, heavily parasitising winter rye in the Sandefjord region. Comparative studies, including morphology, protein variability and virulence pattern, of two Norwegian populations with known Swedish *H. avenae* and *H. filipjevi* populations confirmed the presence of *Heterodera filipjevi* (Madzhidov, 1981) Stelter, 1984 in Norway and constitutes a new geographical record. The pathotype tests demonstrated that the two populations were closest to the Swedish pathotype "West".

Cereal cyst nematodes, *Heterodera* spp., have been recognised for many years in several parts of the world, e.g. Australasia, India and Europe, including Norway (Meagher, 1982; Rivoal and Cook, 1993; Evans and Rowe, 1998; Støen, 1971). The most common and important species is *Heterodera avenae.* The "*Heterodera avenae*-complex" (Stone and Hill, 1982) in its broadest perspective now includes *H. arenaria* Cooper, 1955; *H. avenae* Wollenweber, 1924; *H. aucklandica* Wouts and Sturhan, 1995; *H. australis* Subbotin *et al.,* 2002; *H. bifenestra* Cooper, 1955; *H. filipjevi* (Madzhidov, 1981) Stelter, 1984; *H. hordecalis* Andersson, 1975; *H. latipons* Franklin, 1969; *H. mani* Mathews, 1971; *H. pratensis* Gäbler *et al.*, 2000; *H. spinicauda* Wouts *et al.,* 1995; *H. ustinovi* Kirjanova, 1969 (synonym *H. iri* Mathews, 1971). The virulence status towards cereal cultivars differs between and within species of the *H. avenae* complex, and several pathotypes have been recognised. Borderlines between species and pathotypes often appear unclear.

A survey, carried out during the years 1995-1999 in Norway, revealed that nematodes of the genus *Heterodera* are common throughout the country. Nematodes belonging almost exclusively to the *H. avenae* complex were recorded from the county of Agder in southern Norway (58.08° N) to the county of Nordland in the north (65.5° N) (Holgado *et al.*, 2003). In some cases, nematodes differing from *H. avenae* but similar to *H. filipjevi* were found. This study was undertaken in order to determine the identity and to characterise the pathotypes of these nematodes.

MATERIALS AND METHODS

Soil samples were collected from two different farms near Sandefjord in the county of Vestfold in southern Norway (numbered 184 and 185, respectively). The 184 population came from a field under cereal monoculture. At the time of sampling winter rye, cultivar Danko, was growing in the field and damage was apparent. The two Norwegian populations were subjected to morphological and biochemical studies, and biotests to determine the pathotype. They were compared with the Swedish populations, Etelhem, Skägg and Norra Härene, which belong to *H. filipjevi* (Andersson, 1973; Ferris *et al.,* 1989; Ireholm, 1990, 1994; Valdeolivas and Romero, 1990; Subbotin *et al.,* 1996; Andrés *et al.*, 2001), and the Swedish population, Ask, belonging to *H. avenae* pathotype Ha 11 (Ireholm, 1990).

Extraction of cyst and cyst contents. Soil samples were kept at +4 °C. Before extraction, the soil was air-dried and passed through a 5 mm sieve. Cysts were extracted from 250 g sub-samples by means of a fluidising column (Trudgill *et al.*, 1973). The numbers of *Heterodera* cysts and numbers of viable eggs and juveniles were estimated by standard methods (Southey, 1986; Shepherd, 1986).

Morphological studies. For morphological studies and species identification, mature cysts containing eggs and fully developed juveniles were used. Vulval cones were prepared from mature cysts, and permanent microscope slides were made of second-stage juveniles, fixed in TAF and processed to glycerine (Hooper, 1986). The morphological studies were made using a binocular dissecting Leica M 10 microscope, and a compound Leitz DMR microscope, connected to the Leica Q500MC "Image Processing Analysis System". Air-dried specimens of cyst cones were also prepared for scanning electron microscopy (SEM). The cones were mounted on stubs with the top uppermost, and coated with gold (Southey, 1986) before examination in a Philips XL40 SEM.

Electrophoresis studies. Isoelectric focusing was performed according to principles given by Westermeier (1993), and using the Pharmacia Phast System. Cysts from all the populations were hand-picked into a cavity glass block containing distilled water. They were washed in distilled water several times, and approximately 25 cysts were transferred into a centrifuge tube containing 30 µl of distilled water and frozen at -80 °C until needed.

Protein preparations were made from 25 cysts, which were soaked overnight in 1% glycerol and then homogenised in 25 µl of 1% glycerol using Bio-medix plastic homogenisers. The homogenates were centrifuged for 30 seconds at maximum speed (15,000 *g*) in an Eppendorf 5414 centrifuge, and 10 µl of the supernatant was extracted and transferred to clean, labelled Eppendorf tubes and stored on ice ready for use; 3 µl of the homogenate were then placed in wells pre-formed in Parafilm. From these wells 0.5 µl of the homogenate was taken up by capillary action into the grooves on the Phast System sample applicators.

Each population was tested in triplicate. The proteins profiles on the gels were interpreted from markers run at either side of each gel. Gels of pH range 3-9, supplied by Pharmacia, were used. The mini-gel images were scanned to a computer after which the SigmaGel (Jandel Scientific, California, USA) program was used to analyse the data.

Pathotype test. The tests were performed with techniques similar to those described by Håkansson and Videgård (1966) and Ireholm (1990). Plastic bags (90 x 150 mm transparent polyethylene) were filled with 75% sand and 25% soil (a total of 300 g) and placed in a box (50 bags/box). Eggs were hatched from cysts in distilled water, and an inoculum of 5-10 juveniles per gram of soil was used for testing. A pre-germinated seed was planted in each bag. The boxes were kept in a glasshouse at 15-18 °C with a 16-hour day length. Ten weeks after planting the roots were washed and the visible white females were counted. The cultivars used in this study were selected from an established international test assortment (Holm Nielsen, 1972; Andersen and Andersen, 1982).

The first pathotype test was carried out in 1999 and the second in 2000. In the first test (Table II) the four barley differentials (resistance genes in brackets) Varde (Rha) (control), Emir (Rha "E"), Ortolan (Rha1) and Morocco C.I. 3902 (Rha3) (Andersen and Andersen, 1970; 1982), and the oat cultivar Nidar II were used. The second test (Table III) included the same assortment as above, but was supplemented with the oat cultivar Hedvig and the wheat cultivars Prins and Loros in order to confirm the absence of *H. avenae* pathotypes Ha 11, Ha 12 and Ha 51. A plant was considered resistant if the number of females was less than 5% of the mean value of the control Varde.

RESULTS

Morphological studies. Clear morphological differences were observed between the Swedish *H. avenae* population Ask, and the *H. filipjevi* populations Skägg, Norra Härene, and Etelhem. The two Norwegian populations 184 and 185 were very similar to the three *H. filipjevi* populations from Sweden.

Morphological observations on the juveniles (Holgado *et al.*, 2004) of the Norwegian populations 184 and 185, and of the Swedish populations Etelhem and Norra Härene also showed that juvenile characters closely agreed with those of *H. filipjevi* from Tadjikistan and other populations from the former USSR (Madzhidov, 1981; Subbotin *et al.*, 1996).

The following cyst characters that were observed are (summarized in Table I): Cyst colour: *Heterodera avenae* cysts have a dark brown to black cuticle, while *H. filipjevi* cysts and cyst of the Sandefjord populations have a golden to light warm brown cuticle. In these populations eggs could easily be observed through the cyst cuticle.

Cyst cone. Heterodera filipjevi cones and the cones of the Norwegian populations have a well developed underbridge, whereas the underbridge of *H. avenae* is weakly developed or absent. Bullae in *H. avenae* are strong, dark brown, numerous, distinct and variable in shape, whereas bullae in the Swedish *H. filipjevi* populations and the Norwegian material are weak to medium, distinct, mostly globular, and pale to medium brown in colour. The semifenestrae of *H. avenae* are oval to circular (Fig. 1A), whereas semifenestrae in the Norwegian populations are horseshoe-shaped (Fig. 1B and C). This is also true for the *H. filipjevi* populations from Sweden.

Electrophoresis studies. The positions of the marker protein bands isoelectric points (pI) and the population banding patterns are shown in Fig. 2. The Sandefjord populations have a very similar protein profile to the *H. filipjevi* population Etelhem, with a major band at pI 6.0 that is not found in the *H. avenae* Ask population (arrowed in Fig. 2). The latter differs from the other populations by having a band at pI 5.8.

Pathotype test. The results of the two pathotype tests are given in Tables II and III, and the host status of cultivars to the populations of *H. filipjevi* is presented in Table IV. The numbers of white females produced by the Sandefjord population 184 on the differential barley cultivars in the two tests were on both tests: 0% and 31.2% on cultivar Emir, 3.8% and 0% on cultivar Morocco, and 0.1% and 0% on cultivar Ortolan, respectively, when compared with the numbers of females produced on cultivar Varde (Tables II and III). The numbers of white females produced by the Sandefjord population 185 on the differential barley cultivars were: 82% on cv. Emir, 0% on cv. Morocco and 0% on cv.

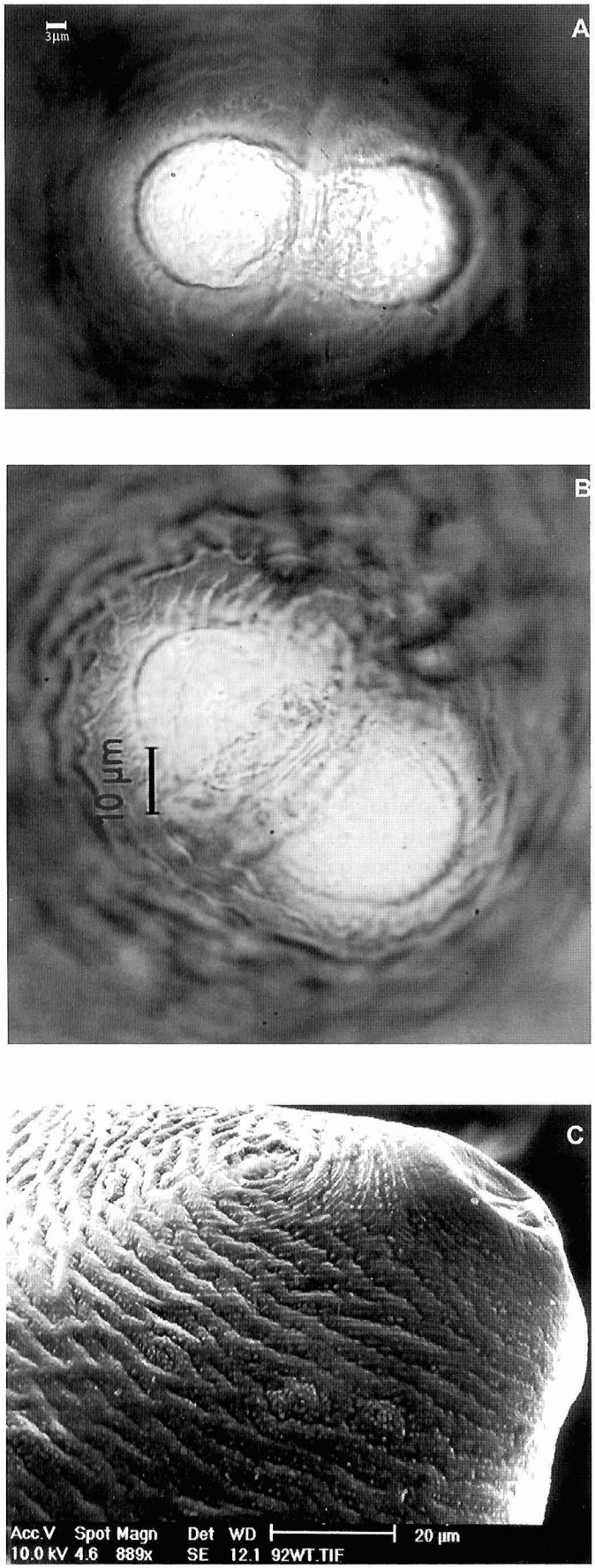

Fig. 1. Vulval cone of *Heterodera avenae* (A) showing an oval, almost circular semifenestra, and vulval cone of *Heterodera filipjevi* (B and C) (pop 184) Sandefjord, showing a horseshoe-shaped semifenestra.

Ortolan, when compared with the numbers on cultivar Varde (Table III). All populations tested appeared to be avirulent to the oat cultivar Hedvig, but virulent to the oat cultivar Nidar II (Tables II and III). All populations reproduced on the wheat cultivars Prins and Loros (Table III). The Sandefjord populations, proved to be moderately virulent to the two wheat cultivars, whereas populations Norra Härene and Etelhem showed a higher degree of virulence (Table III).

On preparing the cysts for hatching, differences in hatching times were observed. The populations from Skägg, Norra Härene, Etelhem and Sandefjord hatched almost spontaneously after cyst extraction, whereas the population Ask did not begin to hatch until at least 24 hours had passed.

DISCUSSION

Morphological studies on the two Sandefjord populations and the Swedish *H. filipjevi* populations Etelhem, Skägg and Norra Härene showed that cyst characters agree closely. This was also true when comparing these populations with published information on *H. filipjevi* from Tadjikistan and other populations from the former USSR (Madzhidov, 1981; Subbotin *et al.*, 1996).

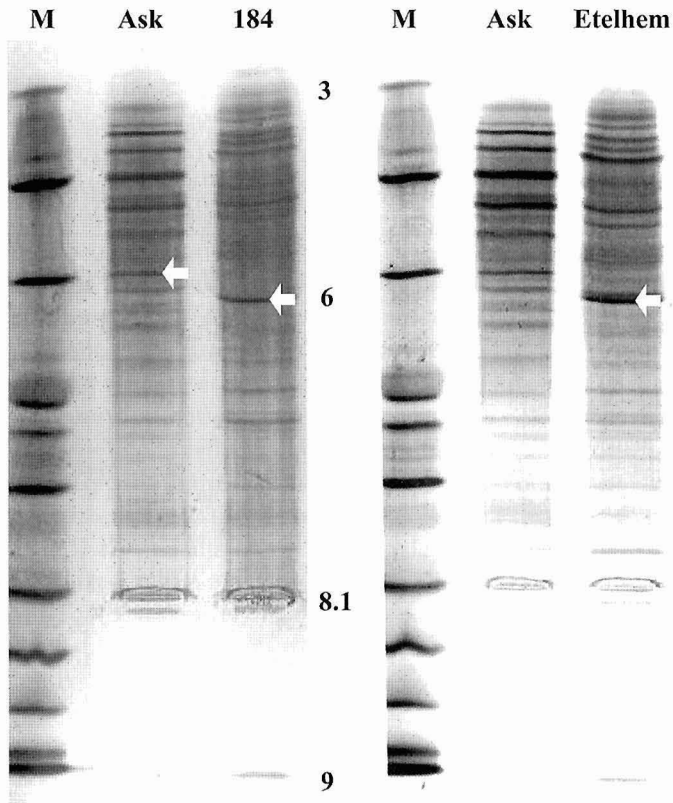

Fig. 2. Comparison of *Heterodera* populations, *H. avenae* (tracks Ask) and *H. filipjevi* (tracks 184 and Etelhem) by isoelectric focusing of water-soluble proteins in the pH range 3-9 on Pharmacia PhastSystem followed by silver staining (M = reference pI marker tracks).

Table I. Morphological differences and similarities between Ask (*Heterodera avenae*) and Skägg, Norra Härene, Etelhem (*H. filipjevi)* and Sandefjord populations.

Population	Fenestration	Bullae	Vulval slit	Underbridge	Cyst colour
Ask (Sweden)	Bifenestrate, semifenestra oval to almost circular	Strong, dark brown, numerous, distinct and variable in shape	Short	Present or absent, when present poorly developed and delicate	Dark brown to black
Etelhem, Skägg, Norra Härene (Sweden), Sandefjord (Norway)	Bifenestrate, semifenestra horseshoe-shaped	Present, weak to medium, distinct and mostly globular, colour pale to medium brown	Short	Present	Golden to light warm brown, eggs generally readily visible through cyst cuticle

Table II. Mean and range of variation of white females on differentials tested against the Sandefjord population and *H. filipjevi,* pathotype "East". First test, 1999.

Cultivar	Pop. 184 Sandefjord (Norway)			Pop. Skägg "East" (Sweden)		
	No. of plants	Mean (%)	Range	No. of plants	Mean (%)	Range
Barley						
Varde	4	25.7 (100)	20-31	4	74.5 (100)	52-102
Emir	4	0 (0)		4	46.2 (62)	35-58
Ortolan	4	0.2 (0.1)	0-1	4	30 (40)	11-42
Morocco	4	1 (3.8)	0-2	4	2.25 (3)	1-4
Oats						
Nidar II	4	26 (100.9)	18-35	4	59,5 (79.8)	30-92

Table III. Mean and range of variation of numbers of white females on differentials tested against the Sandefjord populations and two *H. filipjevi* populations (Etelhem, "East" and Norra Härene, "West"). Second test, 2000.

Cultivar	Pop. 184 Sandefjord (Norway)			Pop. 185 Sandefjord (Norway)			Pop. Etelhem "East" (Sweden)			Pop. Norra Härene "West" (Sweden)		
	No. of plants	Mean (%)	Range	No. of plants	Mean (%)	Range	No. of plants	Mean (%)	Range	No. of plants	Mean (%)	Range
Barley												
Varde	4	17 (100)	11-21	4	28 (100)	18-36	4	44.8 (100)	18-66	2	86 (100)	83-89
Emir	4	5.3 (31.1)	3-9	4	23 (82)	11-28	4	21.2 (47)	16-24	4	0.5 (0.5)	0-2
Ortolan	4	0 (0)		4	0 (0)		3	6.6 (14.7)	2-11	4	0 (0)	
Morocco	4	0 (0)		4	0 (0)		4	1.0 (2.2)	1-2	4	1 (1.1)	0-2
Oats												
Nidar II	4	38 (223)	30-48	3	64 (228)	56-78	4	29 (64.8)	19-37	4	46 (53.4)	36-54
Hedvig	4	0.7 (4.4)	0-2	4	0.2 (0.9)	0-1	4	0 (0)		3	0 (0)	
Wheat												
Prins	4	18 (105)	14-19	4	19 (67.8)	11-29	4	56.2 (125)	30-79	4	53 (61.6)	32-72
Loros	4	2.7 (16)	2-4	4	3.7 (13.3)	1-8	4	77.5 (173)	46-92	4	68.5 (79.6)	42-82

Table IV. Host status of cultivars for populations of *H. filipjevi*. S = susceptibility, R = resistance, (R) = moderate resistance, (S) = moderate susceptibility and (–) = not tested.

Cultivar	*H. filipjevi* "West"*	*H. filipjevi* "East"*	Pop. 184 Sandefjord (Norway)	Pop. 185 Sandefjord (Norway)	Pop. Norra Härene "West" (Sweden)	Pop. Skägg "East" (Sweden)	Pop. Etelhem "East" (Sweden)
Barley							
Varde	S	S	S	S	S	S	S
Emir	R-(R)	(S)-S	S	S	(R)	S	S
Ortolan	R	S	R	R	R	S	S
Morocco	R-(R)	R-(S)	R	R	R	(S)	(S)
Oats							
Nidar II	S	S	S	S	S	S	S
Hedvig	R	R-(S)	R-(R)	R-(R)	R	-	R
Wheat							
Prins	S	S	S	S	S	-	S
Loros	(R)-S	(R)-S	(R)-S	(R)-S	S	-	S

* After Ireholm (1990)

This also applied to juvenile characters. The cyst characters, such as cyst colouration, the weak to medium development of bullae, the presence of a distinct underbridge, and the horseshoe-shaped semifenestra, have already been mentioned for the "Gotland" strain of *H. avenae* by Andersson (1973). In the present study, all these characters could be verified and were found to be useful for the identification of *H. filipjevi*.

The differences in the IEF position of the isoelectric points and the banding patterns clearly allowed us to separate the Swedish *H. filipjevi* population Etelhem and the Norwegian Sandefjord populations from *H. avenae*. IEF has previously been employed to separate nematode species (Karssen *et al.,* 1995; Ibrahim and Rowe, 1995; Hooper *et al.*, 1999). Several protein bands were found in common between *H. avenae* and *H. filipjevi*, indicating a close relationship. This situation is similar to that of other closely related species, like *Globodera rostochiensis* and *G. pallida* (Fleming and Marks, 1983). The results are in line with previous studies of protein patterns from populations of cereal nematodes attributed to *H. avenae sensu stricto* and to the "Gotland" strain (Ferris *et al.,* 1989, 1994; Rumpenhorst *et al.*, 1996). IEF is not sensitive enough to diagnose pathotypes in the populations studied, which also agrees with earlier studies on cereal cyst nematodes (Ferris *et al.*, 1986; Bakker and Bouwman-Smits, 1988).

The cultivars Emir, Morocco and Ortolan should discriminate between *H. filipjevi* pathotypes, all of them being reported resistant to pathotype "West" and susceptible to pathotype "East" (Ireholm, 1985, 1990, 1994). Of the two oat cultivars, cv. Nidar II is susceptible to *H. avenae* pathotypes Ha11 and Ha12, and to both the *H. filipjevi,* pathotypes, while cv. Hedvig is resistant to both *H. avenae* and *H. filipjevi* (Ireholm 1985, 1990). Of the two wheat cultivars, cv. Prins is susceptible to both *H. avenae* and *H. filipjevi*, while cv. Loros is resistant to the *H. avenae* pathotypes Ha11 and Ha12, and moderately resistant to *H. filipjevi* pathotype "West" (Ireholm, 1985, 1990, 1994).

Our results allowed us to reject the possibility of the presence of the *H. avenae* pathotypes Ha 11, Ha 12 and Ha 51. The study also shows that the Swedish population Norra Härene belongs to pathotype "West" while the populations Skägg and Etelhem were designated pathotype "East". This is consistent with previous studies (Ireholm, 1990, 1994). However, in the first test the Norwegian population 184 was avirulent to the cultivars Emir, Ortolan, and cv. Morocco, while in the second test both the Norwegian populations 184 and 185, were virulent to cv. Emir but not to cvs. Ortolan and Morocco. This indicated that in their reproductive behaviour the two populations resemble pathotype "West" of *H. filipjevi* in all respects except that they proved to be virulent on the barley cultivar Emir. Pathotype "West" is reported as avirulent to this cultivar (Ireholm, 1990, 1994). The results of the pathotype test suggest that cv. Ortolan could be a more reliable differential test cultivar for both the *H. filipjevi* pathotypes than cv. Emir. The suggestion of virulence in population 184 to the cultivars Emir, Morocco and Hedvig indicates that the pathotype situation of Norwegian *H. filipjevi* populations needs further study. Compared to the Swedish populations, the Norwegian populations of *H. filipjevi* seemed to be less virulent to the tested wheat cultivars. This indicates a degree of variability in virulence within Scandinavian populations.

In conclusion, the morphological data and the IEF data confirmed the occurrence of *H. filipjevi* in Norway, which is a new geographical record. The pathotype tests

also support this identification and indicate that the Sandefjord populations are most similar to pathotype "West" of *H. filipjevi*.

Heterodera filipjevi may cause significant damage to cereals in Norway, but so far this is the first report on *H. filipjevi* causing damage to winter rye.

The rapid egg hatch observed in the laboratory suggests that field population densities could easily be underestimated if based on cyst extractions only. Hence accurate assessments of pre-plant densities probably also require quantification of infective juveniles in soil.

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