RESEARCH REPORT
PORK SAFETY

Title: Survival of North American Genotypes of *Trichinella* in Frozen Pork – **NPB # 07-088**

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Industry Summary:

We have examined the North American genotypes of *Trichinella* (*T. spiralis* (T-1), *T. nativa* (T-2), *T. pseudospiralis* (T-4), *T. murrelli* (T-5), and *Trichinella* (T-6)) for susceptibility to freezing in pork using established parameters for control of *T. spiralis*. Pig infections with North American genotypes of *Trichinella* were established by oral inoculation of infective larvae in 3 month old pigs of mixed sex and breed. Infected pigs were humanely sacrificed 60 days following infection. Muscles from the tongue, masseters, diaphragm, triceps, hams, neck, rump, and loins were ground, pooled, and mixed to assure even distribution of larvae in tissue samples. Worm burdens in collected tissues were assessed by pepsin-HCl digestion. Pork samples containing each species/genotype were chilled by placing 20 grams of each sample in heat-sealable pouches and pressing to a uniform thickness of 2mm, then transferred to a constant temperature refrigerant bath and maintained according to the time temperature combinations described. Pork samples were removed and thawed in a 5^{o}C bath prior to analysis by digestion. Larvae recovered by digestion of cold treated pork samples were inoculated into mice to determine larval infectivity. Results demonstrated that the freezing parameters described for *T. spiralis* (T-1) in pork products are sufficient to render pork safe with respect to *T. nativa*, *T. psuedospiralis*, *T. murrelli*, and *Trichinella* T-6. *Trichinella nativa* and *Trichinella* T-6 have extremely low

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infectivity for domestic pigs, and where that risk remains, freezing pork at proscribed temperatures is sufficient to destroy these larvae. Further, demonstrating the effectiveness of freezing methods against *T. murrelli,* the most common sylvatic genotype in the U.S., and *T. psuedospiralis,* closes this gap in our current knowledge. Demonstration of susceptibility to currently used freezing methods should eliminate concern about the safety of frozen pork products from these *Trichinella* species in pork.

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Scientific Abstract:

We have examined the North American genotypes of *Trichinella* (*T. nativa* (T-2), *T. pseudospiralis* (T-4), *T. murrelli* (T-5), and *Trichinella* (T-6)) for susceptibility to freezing in pork using established parameters for control of *T. spiralis*. Pig infections with North American genotypes of *Trichinella* were established by oral inoculation of 10,000 infective larvae in 3 month old pigs of mixed sex and breed. Infected pigs were humanely sacrificed 60 days following infection. Muscles from the tongue, masseters, diaphragm, triceps, hams, neck, rump, and loins were ground, pooled, and mixed to assure even distribution of larvae in tissue samples. Worm burdens in collected tissues were assessed by pepsin-HCl digestion. Pork samples containing each species/genotype were chilled by placing 20 grams of each sample in heat-sealable pouches and pressing to a uniform thickness of 2mm to assure consistent freezing and thawing rates between samples. Pork samples were transferred to a constant temperature refrigerant bath and maintained according to the time temperature combinations described. Pork samples were removed and thawed in a 5^{o}C bath prior to analysis by digestion. Larvae recovered by digestion of cold treated pork samples were inoculated into mice to determine larval infectivity. Results demonstrated that the freezing parameters described for *T. spiralis* (T-1) in pork products are sufficient to render pork safe with respect to *T. nativa*, *T. psuedospiralis*, *T. murrelli*, and *Trichinella* T-6. *Trichinella nativa* and *Trichinella* T-6 have extremely low infectivity for domestic pigs, and where that risk

remains, freezing pork at proscribed temperatures is sufficient to destroy these larvae. Further, demonstrating the effectiveness of freezing methods against *T. murrelli*, the most common sylvatic genotype in the U.S., and *T. psuedospiralis*, closes this gap in our current knowledge. Demonstration of susceptibility to currently used freezing methods should eliminate concern about the safety of frozen pork products from these species in pork.

Introduction:

For much of the 20th century the consumption of fresh pork in the domestic market, and the export of pork and pork products, suffered from a negative image derived from the historical presence of *Trichinella* in U.S. pigs. Over the past 50 years, changes in the U.S. pork industry have all but eliminated *Trichinella* as a risk to consumers of U.S. pork. However, documentation of pork safety relative to this parasite has been lacking and therefore the process of gaining consumer confidence in the domestic market and accessing new export markets has been slow.

The U.S. has relied on two strategies for protecting public health relative to *Trichinella* in pork. The first is education of consumers regarding the need to cook pork and pork products thoroughly. The second is treatment of all ready-to-eat pork products by methods that have been scientifically proven to inactivate the parasite (Kotula et al., 1983; Kotula et al., 1990). These processing methods include cooking, curing, and freezing, and are described in the U.S. Code of Federal Regulations (9CFR 318.10). International market access for frozen pork has been limited and trading partners frequently require individual carcass testing in addition to cold treatment. In recent negotiations, trading partners agreed to accept frozen pork from the U.S. as part of the WTO agreement http://www.ustr.gov/assets/Document_Library/Fact Sheets/2006/asset_ upload_file991_9978.pdf). This agreement was reached in spite of objections raised by international veterinary experts regarding a risk posed by cold tolerant species of *Trichinella*, some of which are found in the U.S. and Canada (Pozio, 2001a; Hill et al., 2005). The recent interest in issues surrounding international standards for the cold treatment of pork led members of the International Commission on Trichinellosis to review the current status of the problem and point out the need for additional research on this topic (http://www.eurosurveillance.org/ew/2006/061116.asp).

The research that forms the basis for the current cold treatment requirements for pork (9CFR 318.10; EU

Commission Regulation 2075/2005; Gamble et al., 2000) was conducted in 1990 and was designed to determine the time and temperature combinations that inactivate *Trichinella spiralis* (T-1) in pork. While the cold tolerant species *Trichinella nativa* (T-2) was known to exist, its possible role in pig infections and human disease was not considered, and the existence of 3 other species and genotypes of *Trichinella* in North America was not known. These include *T. pseudospiralis*, *T. murrelli* (T-5), and *Trichinella* T-6, all found in North American wildlife. *Trichinella* T-6 is known to be resistant to freezing, while the cold tolerance of *T. murrelli* and *T. pseudospiralis* is not clear. Studies suggest that the ability to resist freezing is dependent on a variety of factors, including the host (Pozio et al., 1994). For example, *T. spiralis* shows some tolerance to cold temperature in aberrant species such as the horse, and *T. nativa* may be more susceptible to cold treatment in pork (Kapel et al., 2004). However, these preliminary studies require confirmation. The lack of information on how established methods for cold treatment of pork affect species of *Trichinella* other than *T. spiralis* leaves a gap in knowledge that can be used to limit trade. Further, the possibility that a human infection could result from pork that had been treated by established methods, due to the presence of a cold tolerant species or genotype, would have serious negative effects on the further use of freezing as a mitigation strategy. For these reasons, a complete understanding of the effect of cold treatment on all relevant species of *Trichinella* is needed to assure this method is used effectively. The presence of sylvatic isolates in North America which are freeze tolerant is of concern to our trading partners, and represents a barrier to increased trade of frozen pork products. This purpose of the current study was to fill the current gap in knowledge about susceptibility to cold treatment of sylvatic isolates of *Trichinella* which occur naturally in North America, and increase confidence in the strategies currently used to eliminate the risk of *Trichinella* infection from frozen pork.

Objectives:

We will examine the North American species of *Trichinella* (*T. murrelli* (T-5), *T. nativa* (T-2), and *Trichinella* T-6) for susceptibility to freezing in pork. While *T. nativa* and *Trichinella* T-6 have low infectivity for domestic pigs, a low level of risk remains, especially in young pigs, and demonstration of susceptibility to currently used freezing methods will eliminate concern about the safety of frozen pork products from these species in pork. Further, demonstrating the effectiveness of freezing methods against *T. murrelli*, the most

common sylvatic genotype in the U.S., will close this gap in our current knowledge. This research is of interest to Canada as well as the U.S. The research proposed here will be conducted as a collaborative study with the Canadian Food Inspection Agency.

Materials & Methods:

Trichinella species endemic in North America were maintained in the laboratories of the USDA, Agricultural Research Service in Beltsville, MD, USA. Parasites were maintained in Swiss-Webster mice and Sprague-Dawley rats and were recovered and used to inoculate pigs by established pepsin/HCl digestion methods (Gamble, 1996).

Pig infections with North American isolates of *T. spiralis* (T-1), *T. nativa* (T-2), *T. pseudospiralis* (T-4), *T. murrelli* (T-5), and *Trichinella* (T-6) were established in 3 month old pigs of mixed sex and breed. Three pigs were used for each species/genotype, and each pig was orally inoculated with ~10,000 muscle larvae isolated from infected Swiss-Webster mice or Sprague-Dawley rats. *Trichinella* isolates were initially derived from North American wildlife (T-1, raccoon; T-2, black bear (ISS code 1552); T-4, black vulture (ISS code 470); T-5, coyote (ISS code 1657); T-6, cougar (ISS code 456)).

Infected pigs were humanely sacrificed 60 days following infection and muscles from the tongue, masseters, diaphragm, triceps, hams, neck, rump, and loins were trimmed of excess fat and connective tissue. Tissue specific worm burdens were assessed by pepsin-HCl digestion (Gamble, 1996) of three 1gram samples from each muscle collected, totaling 24 grams from each pig.

Muscle tissue from each pig was individually ground using a commercial Hobart meat grinder, then pooled and mixed to assure a uniform distribution of larvae in pork samples. To determine tolerance to cold treatment, pork samples containing larvae of each species/genotype was chilled according to procedures described by Kotula et al., (1990). Briefly, samples weighing 20 grams each were placed in heat-sealable pouches and pressed to a uniform thickness of 2 mm to assure similar freezing and thawing rates between samples. Bags were evacuated and sealed, then stored at 4^{o}C until used in experiments. For cold tolerance experiments, samples were transferred to a constant temperature refrigerant bath (Polystat, Cole Parmer, Vernon Hills IL) and maintained according to the time temperature combinations described in Table 1 below.

Equipment used for cold treatments was monitored daily to assure constant temperature exposure. Samples were removed from cold treatment and thawed in a 5°C bath prior to analysis.

Five 20 gram packaged pork samples containing larvae of each species were tested at each time and temperature combination listed in current regulatory documents (listed in Table 1; 9CFR 318.10, §c.2).

All cold-treated samples, and a set of non-treated positive control samples for each *Trichinella* genotype, were digested using established methods using pepsin/HCl. Briefly, Pepsin (1%):HCl (1%) digestion (Gamble, 1996) was performed by mixing the cold treated tissue with the artificial digestion fluid warmed to 45°C. The mixture was stirred at 45°C for 3 h, then allowed to settle for 20 min. The sediment containing muscle larvae (ML) was repeatedly washed with 250ml of tap water and allowed to settle until the supernatant was clear. The settled ML were counted on a stereo microscope at (40x) then orally inoculated into two Swiss-Webster mice (500 ML each). In those instances where insufficient numbers of ML were collected, the available ML were equally divided and orally inoculated into two mice. After 35, mice were killed by cervical dislocation, skinned, eviscerated and digested as described above to isolate and enumerate ML.

In addition, 1 set of 5 *T. spiralis* samples was treated and digested along with each genotype to assure that appropriate conditions had been met for the destruction of *T. spiralis* larvae using the conditions proscribed in the federal regulations. Each digested sample was examined for recovered larvae. All larvae (motile or non-motile) recovered from each 20 gram digested sample was tested for infectivity by oral inoculation into 1 Swiss-Webster mouse. A maximum inoculation level of 500 muscle larvae per mouse was given. If fewer than 500 larvae were recovered from the 5 samples from each treatment, all larvae were pooled and inoculated into a minimum of 2 mice. Mice were individually digested 35 days following inoculation and the presence and number of viable *Trichinella* larvae was determined.

Results:

Pigs inoculated with North American genotypes of *Trichinella* became infected, and muscle larvae were recovered from the muscle groups collected; as expected, muscle larvae burden was highest in the predilection sites of the tongue, masseters, diaphragm, and neck muscles (Table 2). However, few larvae were recovered from pork samples infected with either *T. nativa* (T-2) or *Trichinella* T-6. Fewer than 0.4 larvae per gram of

tissue were recovered in the most heavily infected *Trichinella* T-6 tissues (tongue), and only 3 larvae were recovered from the 3 pigs infected with *T. nativa*; 1 pig had no larvae recovered.

Motile and coiled larvae were recovered from control (untreated) 20 gram packaged samples from each genotype tested (Table 3). All recovered larvae were orally inoculated into mice, and the mice were killed and digested 35 days post inoculation. All inoculated mice became infected (except the *T. nativa* inoculated mice), and muscle larvae were recovered by digestion from each inoculated mouse 35 days post infection.

No motile larvae were recovered from any cold treated sample for any *Trichinella* genotype tested except for those samples treated at +20 ^{o}F. Recovered larvae were uncoiled and non-motile. However, all larvae recovered from cold treated samples from each genotype at each time/temperature permutation were inoculated into mice to determine infectivity. Mice were killed and digested 35 days post inoculation. No larvae were recovered from inoculated mice except those inoculated with worms isolated from samples treated at +20 ^{o}F (Table 4, A-E).

Discussion:

Eight sibling species and three genotypes of undetermined taxonomic status have been identified in the genus *Trichinella* (Kapel, 2000; Murrell et al., 2000; La Rosa et al., 2003; Pozio and Zarlenga, 2005). Worldwide geographic distribution of these isolates has been described (Kapel, 1997; Pozio, 2001a,b; Zarlenga et al., 2006). Recent studies have shown that 5 of the sibling species (*T. spiralis* (T-1), *T. nativa* (T-2), *T. pseudospiralis* (T-4), *T. murrelli* (T-5), and *Trichinella* (T-6)) occur in North America, and 2 of these sibling species, *T. nativa* and T-6, are capable of surviving for extended periods of time in frozen muscle at temperatures from −5 to −18°C (Kapel et al., 1999; Malakauskas and Kapel, 2003; Hill et al., 2005). Human trichinellosis caused by *Trichinella spiralis*, one of the non-freeze tolerant species, has historically been linked to the consumption of raw or undercooked pork or certain game meats (e.g., bear, wild boar), and much effort has gone into protecting consumers of pork from exposure to *T. spiralis* through carcass inspection, consumer education campaigns describing proper cooking temperatures (Gamble et al., 2000; Webster et al., 2006), and in the U.S., mandatory treatment of fresh pork by freezing or salting if it is not intended to be cooked (Federal Register, CFR 318.10). Concerns about the safety of frozen pork have been raised given the occurrence of the

freeze tolerant genotypes in North America, as well as the uncharacterized ability of other *Trichinella* species to survive in frozen pork.

Food safety requirements dictated for the treatment of fresh pork products detail specific time and temperature procedures for cold treatment to eliminate the risk of infection with *T. spiralis* to consumers. Results from the present study have demonstrated that the current regulations governing treatment of pork products by freezing for the purpose of inactivating *Trichinella* are sufficient for inactivation of all *Trichinella* species which exist in North America. Further, the results demonstrate that there is minimal risk to pigs of infection with *T. nativa* and *Trichinella* T-6, the freeze-tolerant genotypes, since these genotypes have very low infectivity for pigs.

Demands of consumers and trading partners for pathogen-free meat products have focused attention of government regulators and the meat industry on food safety, and the necessity to produce meat that is wholesome, safe and of high quality. Research which demonstrates the safety of meat treated by currently accepted methods such as freezing improves the image of pork products to consumers and trading partners, and will impact the swine industry by increasing trade opportunities for pork products.

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Table 1:

Degrees ^{o}F	Degrees ^{o}C	Minimum Time
+20	-6.6	106 hrs, 53 hrs, 26.5 hrs
0	-17.8	106 hrs, 53 hrs, 26.5 hrs
-5	-20.6	82 hrs, 41 hrs, 20.5 hrs
-10	-23.3	63 hrs, 31.5 hrs, 15.75 hrs
-15	-26.1	48 hrs, 24 hrs, 12 hrs
-20	-28.9	35 hrs, 17.5hrs, 8.75hrs
-25	-31.7	44hrs, 22 hrs, 11hrs, 5.5 hrs
-30	-34.5	16hrs, 8 hrs, 4hrs, 2hrs
-35	-37.2	1hr, 1/2 hrs, 15 min, 7.5 min

Table 2:

TRICHINELLA GENOTYPE	TISSUE	**ANIMAL NUMBER** ML burden		
		308	**602**	**603**
T-1 (*spiralis*)	HAM	*1575	100	700
	MASSETER	2625	1187	880
	TONGUE	9810	3100	3360
	DIAPHRAM	2405	1312	935
	TRICEPS	480	350	1050
	RUMP	870	437	600
	NECK	3200	720	1000
	LOIN	570	480	225
		302	**304**	**305**
T-2 (*nativa*)	HAM	0	0	0
	MASSETER	0	0	0
	TONGUE	0	0	0
	DIAPHRAM	0	0	0
	TRICEPS	0	0	1
	RUMP	0	2	0
	NECK	0	0	0
	LOIN	0	0	0
		553	**558**	**562**
T-4 (*pseudospiralis*)	HAM	21	79	63
	MASSETER	372	569	433
	TONGUE	735	766	700
	DIAPHRAM	145	368	540
	TRICEPS	16	460	266
	RUMP	88	200	324
	NECK	226	266	187
	LOIN	33	19	126
		303	**531**	**620**
T-5 (*murrelli*)	HAM	21	3	5
	MASSETER	32	0	0
	TONGUE	112	1	6
	DIAPHRAM	75	8	6
	TRICEPS	13	0	1
	RUMP	22	1	0
	NECK	31	2	0
	LOIN	28	2	3
		525	**604**	**682**
T-6	HAM	0	0	0
	MASSETER	0	0	1
	TONGUE	0	9	1
	DIAPHRAM	0	5	1
	TRICEPS	0	0	1
	RUMP	0	0	0
	NECK	1	1	0
	LOIN	2	0	0

*Total larvae in 3 1-gram samples.

Table 3: Muscle larvae recovery from control (untreated) samples and from mice inoculated with larvae recovered from control samples.

Trichinella genotype	ML recovery from control pork samples, n=5*, mean	ML recovery from infected mice, n=5**, mean
T-1 (*spiralis*)	4303.00	23794.44
T-2 (*nativa*)	2	(n=2)** 0
T-4 (*pseudospiralis*)	1336.00	13012.50
T-5 (*murrelli*)	359.2	2008.40
T-6	15.60	(n=2)** 443.07

*= number of pork samples digested to derive mean number of ML recovered.
**=number of mice digested to derive mean number of ML recovered. Fewer than 100 ML were recovered from T-2 and T-6 pork samples; recovered ML pooled and inoculated into 2 mice.

Table 4(A-E): ML recovery from cold treated samples, n=5*.

A: Results of cold treatment of pork samples containing *T. spiralis* (T-1)

Storage Temp, ^{o}F	Storage Time	Recovered ML	Storage Time	Recovered ML	Storage Time	Recovered ML	Storage Time	Recovered ML
+20	106 hrs	4033/**21,700**	53 hrs	5166/**15,800**	26.5 hrs	2766/**12,500**		
0	106 hrs	0	53 hrs	0	26.5 hrs	1657.4		
-5	82 hrs	1826.4	41 hrs	4169.6	20.5 hrs	1630.6		
-10	63 hrs	2399.8	31.5 hrs	0	15.75 hrs	0		
-15	48 hrs	0	24 hrs	1393.0	12 hrs	526		
-20	35 hrs	0	17.5hrs	526.6	8.75 hrs	3214.8		
-25	44 hrs	1006.2	22 hrs	199.8	11 hrs	891.2	5.5 hrs	212.8
-30	16 hrs	1775.2	8 hrs	286.40	4 hrs	273.0	2 hrs	2381.0
-35	1hr	523.2	1/2 hrs	1112.6	15 min	326.2	7.5 min	1772.4

*= number of 20g pork samples digested to derive mean number of ML recovered. All mice inoculated with these recovered larvae were *Trichinella* negative by digestion 35 days post inoculation **except** mice given ML treated at +20^{o}F, mean mouse ML recovery in **bold**. Recovered ML= mean recovered muscle larvae from 5-20-gram pork samples at specific storage time and temperature permutation.

B: Results of cold treatment of pork samples containing *T. pseudospiralis* (T-4)

Storage Temp, °F	Storage Time	Recovered ML	Storage Time	Recovered ML	Storage Time	Recovered ML	Storage Time	Recovered ML
+20	106 hrs	1066/**11,200**	53 hrs	1233/**8,600**	26.5 hrs	1429/**13,600**		
0	106 hrs	0	53 hrs	14.6	26.5 hrs	5.4		
-5	82 hrs	0.2	41 hrs	0.2	20.5 hrs	0.4		
-10	63 hrs	0.8	31.5 hrs	0.4	15.75 hrs	0.6		
-15	48 hrs	0.2	24 hrs	0.4	12 hrs	0.2		
-20	35 hrs	0.4	17.5hrs	0	8.75 hrs	0.6		
-25	44 hrs	0.8	22 hrs	0	11 hrs	0	5.5 hrs	0
-30	16 hrs	0.4	8 hrs	0.6	4 hrs	0.4	2 hrs	0
-35	1hr	0	1/2 hrs	0.2	15 min	0.8	7.5 min	3

C: Results of cold treatment of pork samples containing *T. murrelli* (T-5)

Storage Temp, ^{o}F	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**
+20	106 hrs	4033/**2250**	53 hrs	5166/**750**	26.5 hrs	2766/**500**		
0	106 hrs	0	53 hrs	0	26.5 hrs	1657.4		
-5	82 hrs	1826.4	41 hrs	4169.6	20.5 hrs	1630.6		
-10	63 hrs	2399.8	31.5 hrs	0	15.75 hrs	0		
-15	48 hrs	0	24 hrs	1393.0	12 hrs	526		
-20	35 hrs	0	17.5hrs	526.6	8.75 hrs	3214.8		
-25	44 hrs	1006.2	22 hrs	199.8	11 hrs	891.2	5.5 hrs	212.8
-30	16 hrs	1775.2	8 hrs	286.40	4 hrs	273.0	2 hrs	2381.0
-35	1hr	523.2	1/2 hrs	1112.6	15 min	326.2	7.5 min	1772.4

D: Results of cold treatment of pork samples containing *Trichinella* T-6

Storage Temp, ^{o}F	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**
+20	106 hrs	73/**0**	53 hrs	95/**210**	26.5 hrs	68/**0**		
0	106 hrs	0.6	53 hrs	0.8	26.5 hrs	1.4		
-5	82 hrs	2.6	41 hrs	1.8	20.5 hrs	2.4		
-10	63 hrs	1.2	31.5 hrs	1.2	15.75 hrs	1.0		
-15	48 hrs	0	24 hrs	0.6	12 hrs	0.6		
-20	35 hrs	0.6	17.5hrs	2.2	8.75 hrs	1.4		
-25	44 hrs	0.4	22 hrs	0.2	11 hrs	0.2	5.5 hrs	0.8
-30	16 hrs	0.6	8 hrs	1.6	4 hrs	0	2 hrs	0
-35	1hr	1.4	1/2 hrs	2.0	15 min	2.0	7.5 min	1.0

E: Results of cold treatment of pork samples containing *T. nativa* (T-2)

Storage Temp, ^{o}F	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**	**Storage Time**	**Recovered ML**
+20	106 hrs	3/**0**	53 hrs	0/**0**	26.5 hrs	1/**0**		
0	106 hrs	0	53 hrs	0.4	26.5 hrs	0		
-5	82 hrs	0.2	41 hrs	0	20.5 hrs	0		
-10	63 hrs	0.6	31.5 hrs	0.2	15.75 hrs	0.2		
-15	48 hrs	0	24 hrs	0	12 hrs	0		
-20	35 hrs	0.4	17.5hrs	0.4	8.75 hrs	0.6		
-25	44 hrs	0.4	22 hrs	0	11 hrs	0.6	5.5 hrs	0.2
-30	16 hrs	0	8 hrs	0.6	4 hrs	0	2 hrs	0
-35	1hr	0.4	1/2 hrs	0	15 min	0	7.5 min	0.2