# THE BACTERIOLOGICAL ANALYSIS OF WINKLES (*LITTORINA* SPP.) AS THEY ARRIVE IN THE MARKET.

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ON 15. i. 1929 Dr Sowden, the Medical Officer of Health for the Borough of St Pancras, notified the Worshipful Company of Fishmongers that nine (9) cases of enteric fever had occurred in his borough during the last week in December, 1928; and that the individuals infected had all consumed winkles purchased on Sundays during November and December from local stall-holders, who in turn had obtained them from Messrs Baxter and Sons of Billingsgate Market on the preceding Saturdays, and (so they stated) boiled them on Sunday mornings in preparation for sale that day.

Dr Sowden very courteously supplied the following details at a later date:

The stall-holders in question were:

(a) Mrs Woods, who stands with her open street stall on Sundays outside the "Buck" public-house, and, as is customary, retails the winkles at so much per "pint" measure.

Although her winkles are derived from Messrs Baxter and Sons, she purchases them through Mr Haley, who is himself the proprietor of a stall standing outside the "Robert Peel" public-house in Queen's Crescent. (It may be noted in passing that none of the enteric cases could be associated with the consumption of winkles from Mr Haley's stall.)

(b) Mrs Sales, whose stall stands outside the "Stag" public-house, Hawley Street. The winkles are purchased by this purveyor through W. Overnell of Billingsgate.

A sample of *live* winkles obtained by the St Pancras Public Health Department on 5. i. 1929, from the stall of one of the vendors (Mrs Woods) referred to above, was submitted to Prof. F. H. Teale of University College Hospital Medical School for bacterioscopical analysis. He reported that there was no evidence of the presence of *Bacillus typhosus* or any other infective bacillus, but that *B. coli* was present to the number of 100 (but not 1000) per winkle; that *B. proteus* was present in large numbers—also *Streptococcus faecalis.* The total number of micro-organisms capable of growing at 22° C. was 2,140,000 per winkle; and at 37° C. 850,000; anaerobic bacteria were also present to the number of ten per winkle.

A sample of *boiled* winkles (from the same vendor) was examined on 7. i. 1929 and in these *B. coli* was present to the number of one (but not ten) per winkle. Numerous *B. proteus* and *Strept. faecalis* were noted. The total number of micro-organisms capable of growing at  $22^{\circ}$  C. averaged 4900 per winkle; and at  $37^{\circ}$  C., 22,000.

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From these results Prof. Teale concluded that:

(a) The winkles were derived from layings subject to undesirable and potentially dangerous contamination.

(b) The boiling process to which the winkles had been subjected was insufficient to render them "safe."

Assuming for the moment that there was in fact a connection between the consumption of winkles and the occurrence of enteric cases between December 24th and 31st (and the nine cases in question apparently did not include any contact cases), most probably the shellfish eaten on December 9th would be those implicated. But allowing for extremes of incubation time (5 to 22 days) it is possible that those consumed on December 2nd, or even on December 16th, might have been the offenders.

In the ordinary course of events the next steps would have been to ascertain the particular foreshore from which the incriminated winkles were obtained, and enquire into the occurrence of typhoid cases in that locality, and the possibility of the pollution of the foreshore by infective dejecta. This procedure, however, was not practicable for the following reasons:

During the first fifteen days of the month of December 1928, the firm of Messrs Baxter and Sons received seventy-two separate consignments of winkles, including thirty-four from Irish, twenty-seven from Scotch and one from Dutch fishermen. A carefully compiled list of the dates of supply and sources of origin showed that the winkles were fished from forty-five separate and distinct localities, distributed as follows:

England	•••	•••	•••	<b>2</b>
Wales		•••		3
Scotland	•••	•••		16
Irish Free	State	•••	•••	<b>23</b>
Holland	•••	•••	•••	1

Further to complicate the enquiry, the fact was elicited that winkles after fishing, if kept in a damp atmosphere (as in the original packing bag and covered with seaweed) retain their vitality and wholesomeness for a considerable period—even up to 14 days—indeed, it frequently happens that a consignment remains in the market for upwards of a week, before it is sold and removed by the retailers. So that the possibility was present that the winkles sold on those three Sundays in December might have been derived not merely from any one but from a mixture of two or more of the twenty-one British or twenty-four foreign gathering grounds.

Under these circumstances it was impossible to trace the exact locality from which the winkles in question had been collected.

Here the matter might have rested had not the Fish and Fisheries Committee, noting that the records of the Company contained only a few isolated analyses of the winkles, decided that further information was desirable concerning:

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(a) The extent of the pollution exhibited by this mollusc on its arrival in the market.

(b) The degree of safety imparted by the cooking process to which it is subjected prior to sale.

That this section of the shellfish trade is of some importance is shown by the gradual increase in tonnage of winkles received at Billingsgate Market during the past five years, viz.:

	Tons	cwt.	qr.
1927	3475	5	<b>2</b>
1928	3590	2	1
1929	3745	14	0
1930	3702	3	<b>2</b>
1931	3847	13	0

The periwinkle, or as it is usually termed, the winkle, represents a genus of small marine vegetarian gasteropods, containing many species. *Littorina littorea*, one of the commonest, is distributed around the British coasts on rocks, between the tidal marks. It is characterised by its substantial shell of few whorls, its closely fitting horny operculum and its nearly circular shell aperture without any siphon notch.

As compared with the oyster or the mussel, the winkle yields about one tenth the amount of fish flesh. This estimate was arrived at after the examination of five batches each of *ten* winkles, in the natural condition, weighed after removal from the shell. The various batches of flesh weighed 9.08, 9.10, 9.13, 10.62, and 10.99 g. respectively. The individual weights are set out in Table I and whilst showing extremes of 0.57 g. and 1.32 g. yielded an average weight per winkle of nearly 1 g. (0.979)—the average oyster or mussel weighing approximately 10 g.

1st series	2nd series	3rd series	4th series	5th series
0.630	0.683	0.570	0.670	0.780
0.676	0.710	0.590	0.780	0.890
0.760	0.760	0.793	0.997	0.908
0.840	0.870	0.880	1.008	1.010
0.880	0.882	0.942	1.050	1.075
0.889	0.890	0.970	1.142	1.170
0.964	0.910	1.047	1.180	1.240
1.100	1.074	1.050	1.207	1.294
1.160	1.120	1.065	1.287	1.305
1.190	1.180	1.230	1.304	1.324
9.089	9.109	9.137	10.625	10.996

Table I. Recording weights of winkles in grammes.

The enumeration of the individual winkles in a number of pint measures gave an average of 100 per pint.

The winkle remains alive and sound for many days after removal from the fishing ground; it is measured and sold wholesale by the bushel, and retailed by the pint or quart; is "cooked" before consumption, and when thus killed is easily extracted from its shell with the help of a pin or similar instrument. It is much esteemed in some quarters as a tea-table delicacy, when it is consumed, on a rough estimate, in quantities of half a pint per person.

## Bacteria in Winkles

In order to arrive at some idea of the extent of the pollution to which this shellfish is commonly exposed, samples of living winkles from widely separated localities were secured from the market as opportunity occurred.

In all, fifty samples were collected and examined. These were derived from different localities around the shores of the British Isles, and were distributed as follows:

England	•••	•••	8 sa	$\mathbf{mples}$	from	7	areas
Wales	•••		1	,,	,,	1	,,
$\mathbf{Scotland}$	•••	•••	17	,,	,,	16	"
Ireland	•••	•••	<b>24</b>	,,	,,	18	,,

Ten of these areas, viz. Brightlingsea (England), Kirkwall, Cockburnspath, Invergordon, Lochmally (Scotland), Galway, Kilrush, Minerstown, Tragalee and Trallee (Irish Free State), which together provided thirteen of the samples, were localities whence Messrs. Baxter and Sons were in the habit of drawing supplies—sixteen of their consignments in December 1928 were drawn from these places.

Of these fifty samples, thirty-eight were taken during the winter months, and the seasonal collection may be grouped thus:

Spring (January-March)		•••	19 sa	mples
Summer (April—June)		•••	<b>2</b>	,,
Autumn (July-September)			10	,,
Winter (October—December)	•••	•••	19	"

The various samples were collected by the Fishmeters of the Company from the bags in which the shellfish arrived at Billingsgate Market, and taken at once to the laboratory. On arrival ten (10) winkles taken at random were withdrawn from the sample and the shells thoroughly washed under a tap of running water. The shells were then broken with aseptic precautions by means of a pestle and mortar, and the flesh extracted with sterile forceps; after which the examination was proceeded with, in accordance with the method to be detailed shortly.

Since the shellfish are cooked *before sale*, careful enquiries were made as to the exact method of cooking employed by the different merchants; so that by applying as close an imitation of the trade process as practicable to the samples received in the laboratory, it might be possible to ascertain what degree of safety was imparted to the shellfish thereby. After collating the result of our enquiries in this direction, the following method of cooking was adopted.

The samples were tied up in a bag of butter muslin and suspended in a pail containing a 3 per cent. solution of common salt (prepared with tap water under conditions of ordinary cleanliness) for 3 hours, to allow for automatic cleansing.

The bag was then removed and plunged in a vessel of water (not saline) already boiling. As soon as the water (temporarily lowered in temperature) had returned to the boiling point the time was noted and 3 minutes later the

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bag with the winkles was removed and plunged into another clean pail full of clean cold salt (3 per cent.) water for 1 hour. At the end of this process ten of the treated—or cooked—winkles were withdrawn haphazard from the bag, the dead fish extracted from the shells by means of a strong stilette, and examined bacteriologically in precisely the same manner as the living winkle.

As is customary in the bacterioscopical analysis of foods in general, a careful search was made for the presence of "microbes of indication," by the use of selective media specially adapted to their demonstration—thus:

Bile salt lactose broth	for the	detection	of B. coli.
Glucose (2 per cent.) peptone broth	1 <b>,,</b>	,,	Strept. faecalis.
Litmus milk	,,	,,	spores of B. welchii.
10 per cent. gelatine	"	"	B. proteus.

The enumeration of these microbes, when present, was accomplished by the use of definite amounts of winkle flesh, varying from an entire winkle to onemillionth of a winkle, for the insemination of a series of tubes of each of the above-named media, premising that the quantities necessary for the fractional parts corresponding to less than 1/10 of a winkle were obtained by the ordinary process of "decimal dilution" of the fluid in which the winkle flesh had been emulsified.

In dealing with such a series as this for each of the various media employed, more than 70 c.c. of fluid (equivalent to the extract from the bodies of more than six entire winkles) was subjected to analysis.

At the same time evidence as to the presence of members of the Salmonella, Typhoid and Dysentery groups was sought by the use of plate cultures upon Wilson's iron bismuth agar and upon nutrose agar.

Finally, the presumptive evidence of pollution with excremental bacteria yielded by growth under these conditions was carefully investigated by means of plate cultures and the subsequent transfer of suspicious colonies to pure culture, for identification by ordinary routine methods.

## METHOD OF EXAMINATION.

The exact technique that has been followed was gradually evolved during some preliminary trials before embarking upon the examination of the fifty samples that form the subject of this communication. In some of the earliest analyses the trituration of the bodies of the shellfish was carried out with the aid of sterilised silver sand, but it was soon found that it was difficult to obtain sufficient fluid for the preliminary platings. Nor was the presence of sand necessary, for with a little practice it became quite a simple matter to disintegrate the bodies of the shellfish thoroughly with pestle and mortar in the presence of comparatively small additions of 3 per cent. saline solution. Again, many different quantities of the fluid extract were tried in an attempt to obtain a closer approximation to the number of bacteria present, before finally deciding on those quanta which are set out in the following method.

### THE EXAMINATION OF WINKLES AND SIMILAR SHELLFISH.

1. Take ten (10) winkles at random from the sample.

2. Extract each fish from its shell by means of a strong stilette and transfer to a sterile mortar.

3. Triturate the bodies of the fish very thoroughly, adding sterile 3 per cent. saline solution to a total amount of 10 c.c. to the contents of the mortar during the process.

Allow the emulsion to stand for a few minutes whilst the debris settles.

Decant the turbid supernatant fluid (O.F.) (original fluid) into a 250 c.c. Erlenmeyer flask (sterile).

(It is now assumed that the entire bacterial content from one winkle is contained in each cubic centimetre of the O.F.)

4. Use 0.1 c.c. of the original fluid (equivalent to 1/10 winkle) to inseminate three nutrose agar plates in series.

5. Prepare two surface plate cultures, each with 0.1 c.c. of the O.F. upon Wilson's iron bismuth agar for the detection of members of the typhoid group or Salmonella.

Incubate these five plates at 37° C. for 48 hours.

6. Add 90 c.c. of the sterile hypertonic saline to the winkle bodies in the mortar and mix thoroughly.

7. Transfer the mixture to a sterile conical urine glass. Protect this receptable by covering it with the half of a petri capsule, and allow it to remain undisturbed for about 10 minutes whilst the debris settles down. Then decant the supernatant fluid into the flask containing the remainder of the original fluid.

It is now assumed that the bacteria contained in every quanta of 10 c.c. of this "secondary" fluid (S.F.) represent the bacteriological flora of an average winkle.

#### Quantitative examination.

8. Prepare decimal dilutions from 1 c.c. of the secondary fluid, thus:

(1) 1 c.c. secondary flu	id + 9 c.c. salt s	solution = A
(2) 1 c.c. A	+ 9 c.c. "	" = B
(3) 1 c.c. B	+9 c.c. "	,, _= C
(4) 1 c.c. C	+9 c.c. "	,, = D.

9. Prepare a series of agar plates containing respectively 1 c.c. of secondary fluid and 1 c.c. from each of the dilutions A, B, C, D. Incubate at  $37^{\circ}$  C. for 48 hours and then enumerate the resulting colonies.

10. Prepare precisely similar set of gelatine plates. Incubate at  $22^{\circ}$  C. for 72 hours and then enumerate the resulting colonies. This set of plates also serves for the enumeration of *B. proteus*.

#### Qualitative examination.

11. A. B. coli. Prepare a set of cultures in bile salt lactose broth thus—using double strength medium in large tubes for the first two—and incubate for 24 hours.

(a)	10 c.c.	secor	ıdary	fluid	! (=	1	winkle)			
(b)	5 c.c.	:	,,	,,	(==	12	winkle)			
(c)	2 c.c.		,,	"	(=	<del>1</del>	winkle)			
(d)	1 c.c.		,,	"	(=-	$\frac{1}{10}$	winkle)			
(e)	1 c.c.	fluid	from	dilu	tion	Α	(see par.	8) =	1/100	winkle
(f)	1 c.c.	"	"	,	,	В		=	1/1000	,,
(g)	1 c.c.	,,	,,	,	,	С		=	1/10,000	,,
(h)	1 c.c.	"	,,	,	,	D		=	1/100,00	0,,
(i)	0·1 c.c.	<b>,,</b>	"	,	,	D		==	1/1,000,0	)00 "

12. B. Streptococci. Prepare a precisely similar set of cultures using 2 per cent. glucose broth instead of bile salt broth.

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13. D. B. proteus. For the recognition and enumeration of this organism the gelatine plates prepared as per paragraph 10 are again utilised.

14. C. Spores of B. welchii. Inoculate into litmus milk

10 c.c. secondary fluid

5 c.c. ,, ,, ,, 2 c.c. ,, ,, ,, 1 c.c. ,, ,, ,,

Heat to 80° C. for 10 minutes in water bath and incubate anaerobically for 48 hours.

The results were somewhat surprising, for apparently the winkle when freshly arrived in the market does not usually harbour any very considerable number of "microbes of indication." Consequently after the first few examinations the enumeration of the total organisms by plate methods was discarded in order to economise material and permit of closer search for excremental bacteria to be carried out by tube tests. The predominant organism was undoubtedly B. proteus-and its near allies. In nearly half of the samples (twenty-four) it was present in numbers in excess of 500 per winkle; and in fifteen of these was actually innumerable. Spores of B. welchii, on the other hand, in no case exceeded ten per winkle; in thirty-nine did not exceed two per winkle, and in two samples, if present, failed to make that presence evident in the litmus milk cultures. In none of the samples could the presence of any members of the Typhoid group or Salmonella group be detected, and only once was a lactose non-fermenter encountered—one of the Morgan group. B. coli was detected in every sample, and when isolated was true to one or other typecommunis or communior. In a few instances both types were present in the same sample. It was present to the number of 1000 per winkle in three samples, and in fifteen more between 100 and 1000 per winkle, but more than half of the samples (twenty-nine) contained ten or less per winkle. In three instances B. coli was associated with colonies of B. lactis aerogenes.

Strept. faecalis was definitely more prevalent than B. coli; it was present in every sample and although only one sample contained 1000 or more per winkle, thirty-three contained between 100 and 1000, and only sixteen samples contained ten Streptococci or less.

The effect of the "cooking" as carried out on the lines already described on small samples in the laboratory was exceedingly satisfactory. Particularly striking was the reduction of the total count; *B. proteus* usually disappeared altogether—it was only detected in sixteen of the cooked samples—in five of these the count varied from 130 down to 20; in the remaining eleven *B. proteus* numbered 10 six times, and 1 five times. Spores of *B. welchii* obviously would not be destroyed by this short exposure at boiling point, but it is interesting to note that their sparsity was such that they were sometimes missed in the cooked sample although present in the corresponding raw sample. *B. coli* was only detected in seven out of the fifty samples, and then only in ones and twos per winkle.

Streptococci also diminished in numbers, but only from three samples did

they disappear completely; in forty-three out of the remainder their numbers were reduced to ten or less per winkle.

The analytical results of the examination of every sample both before and after cooking, together with details as to time of collection and source of origin, are here set out in tabular form (Table II).

In interpreting the tables it must be noted that the number of individual bacteria stated under any particular heading refers to the culture containing the smallest fraction of a winkle in which growth occurred; thus, in the first instance if the laboratory result were written out in full it would run "B. coli present in 1/5 winkle but not in 1/10 winkle," or alternatively, "B. coli present to the extent of two but not five per winkle." B. proteus was enumerated—or not, as the case might be—from actual counts of the colonies developing upon gelatine plates.

### STANDARDS OF CLEANLINESS.

Hitherto there has been no accepted or even suggested standard of cleanliness set up for the periwinkle; but it seems justifiable to consider the results set out in these tables as a basis upon which to erect a tentative standard, utilising—as in the case of other foodstuffs—*B. coli* as the index of pollution.

This organism has proved a reliable indicator in the examination of other molluscs, notably the oyster, in connection with which two standards are of very general acceptance.

The first, the stringent standard (Houston), postulates that the average oyster—as estimated from the examination of a sample of ten oysters mixed together—shall not contain more than 100 *B. coli*. (A lenient standard, which it would be well to discard altogether, placed the *B. coli* content of the average oyster at a point not exceeding 1000.)

This standard is of prime importance, taken in conjunction with a topographical survey, in evaluating the cleanliness of any particular laying; but has the disadvantage from the marketing point of view in that several days are required for the assembling of the necessary data.

The second is the standard set up by the Fishmongers' Company by which *B. coli* must not be present in numbers exceeding 200 per oyster in more than 50 per cent. of the sample. As the information necessary for its application is available within 24 hours, this standard regulates the sale of oysters entering the London Market.

On tabulating the results of winkle analyses already detailed it appears that

0			~		5				
0 s	amples	contained	0 <b>B</b>	0 B. coli per wink					
1	,,	"	1	,,	"				
4	,,	,,	<b>2</b>	,,	,,				
4	,,	,,	<b>5</b>	,,	,,				
<b>20</b>	"	,,	10	,,	,,				
<b>2</b>	,,	>>	20	,,	"				
16	,,	"	100	,,	,,				
3	» <b>;</b>	,,	1000	,,	"				

or in other words that 58 per cent. of the samples contained ten or less  $B. \ coli$  per winkle on arrival in the market.

This would correspond bulk for bulk or weight for weight with 100 B. coli per oyster (the stringent standard). In this country the oyster is usually consumed in the raw state, but the winkle is cooked. Now looking back at the effect of the laboratory cooking upon the winkles, it is clear that a very considerable degree of safety is imparted to the shellfish by the process there described, since B. coli, which is distinctly more resistant to heat than B. typhosus or any of the Salmonella group, was destroyed in 86 per cent. of the samples, and in the 14 per cent. was reduced to ones or twos. But it is equally obvious to all whose duties require them to examine the cooked winkle as it is exposed for sale, that this shellfish invariably contains living B. coli, sometimes in large numbers-a circumstance that compels the conclusion that the shellfish are not, in practice, exposed to the conditions that obtained in the laboratory. In coming to this conclusion it is not necessary to impute intentional inaccuracy to those vendors who described their methods, but rather inexact observation. The object in view when the vendor cooks his or her winkles is the death of the winkle, in order to facilitate its removal from the shell by the purchaser—not the destruction of the contained B. coli; and the fallacy that creeps into the method is probably in timing the cooking process from the moment the shellfish are plunged into the boiling water instead of the moment when the temperature-lowered by the impact of the mass of shellfish-returns to the boiling point. Assuming that this is the correct explanation, many of the Colon bacilli will certainly escape, and, if kept warm and moist in the semicoagulated protoplasm of the shellfish, will increase in numbers.

But in order to ensure that the cooking process shall be more efficiently performed and at the same time to allow some latitude to inexact observers, it would appear sufficient to set up a standard of "not more than twenty  $B.\ coli$  per winkle" whether in the raw state or "cooked"; and to permit the sale for human consumption of such winkles as conform to that standard when examined by the method already set out.

		Winkles (Raw)	(Raw)		,	Winkles (Boiled	(Boiled	
Source	B. coli	Strept. faecalis	B. welchii	B. proteus	B. coli	Strept. faecalis	B. welchii	B. proteus
Miltown Malbay (I)	2	, 10	٦	50	61	~	I	IIN
Ardglass (I)	20	1000	10	400	61	100	10	:
Brightlingsea (E)	100	õ	ľ	8	IIN	ũ	1	10
Minerstown Clough (I)	20	100	67	500	*	100	67	Г
Kilrush (I)	ũ	61	Ð	liN	:		IIN	Nil
Tragalee (I)	61	1	61	œ	:		2	ŝ
Killarney (I)	õ	61	õ	8	:	I	*	:
Miltown Malbay (I)	100	õ	61	8	:	61	۲	2
Brightlingsea (E)	10	5	63	Nil		I	IIN	2
Ardglass (I)	10	10	67	150		1	1	10
Ardglass (I)	100	100	<b>61</b>	500	-	67	IIN	70
Lochmaddy (S)	10	100	67	I	67	-	I	Nil
Invergordon (S)	100	100	5	8	liN	67	61	10
Cockburnspath (S)	100	ũ	5	450	:	IIN	I	09
Kirkwall (S)	100	100	61	510	:	63	61	30
Belfast (I)	ũ	100	61	30	2	5 CI	IIN	Nil
Cork (I)	100	100	\$1	50	:	10	:	I
Tralee Bay (I)	100	10	67	8	67	õ	61	I
Sth Queensferry (S)	100	100	61	8	I	10	I	I
Maidens (S)	100	100	I	8	I	10	1	I
Inver-Fearn (S)	61	õ	61	30	Nil	63	IIN	20
Nr. Filey (E)	100	100	õ	8	:	ũ	I	10
Caernarvon Bay (W)	1000	100	7	8	:	100	I	130
Broadford (S)	10	100	10	8	:	I	IIN	10
Carrigalin (I)	10	100	1	500	:	ŝ	:	liN
Rainham Creek (E)	10	100	67	Nil	:	10	2	:
Galway Bay (I)	100	100	5	8	:	10	:	:
Donegal Bay (I)	10	100	ũ	100	:	61		:

Bacteria in Winkles

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			: :	5 5	: :	: :	: :	: :		r 1	=	(W) = Wales.
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ntinued	100 100	001	001	100	100	001	100	01 P	2 00 I	2 00 1 00 1	100	(S)
Table II (continued)	1000 1000	2 0 Q	100	1001	10	10	01	10	10	100	10	(I) = Irish Free State.
	Dunbar (S) Donegal (I) Castlemartyr (I)	Glenburgh (I) Lympstone (E)	Galway Bay (I) Truro Bay (E)	Dunure (S) Eyemouth (S)	Fethard (I) Bantry Bay (I)	Tranmore (I) Fraserburgh (S)	Fife (S) Johnshaven (S)	Carbost (S) Carrigart (I)	Shanagarry (I) Ardglass (I)	Fraserburgh (S) Southend (E)	Chapman Sands (E)	(E) = England. (I) = Lris
	Nov. 5 ,, 7 ,, 13	""" "21	,, 25 ,, 28		" 12 " 18	" 19 Jan. 7	,, 13 ,, 15	, 21 , 23	., 29 Feb. 5	" 6 " 10		(E)=
	2036 2041 2049	2050 2058	2059 2063	2073 2074	2077 2087	2089 2101	2103 2106	2111 2112	2133 2150	2153 2166	2167	

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