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Comparison of media and methods for counting *Clostridium perfringens* in poultry meat and further-processed products

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SUMMARY

A Most Probable Number (MPN) method involving Differential Reinforced Clostridial Medium followed by streaking on Willis & Hobbs medium was compared with direct plating of samples on Tryptose-Sulphite-Cycloserine agar without egg yolk, and two forms of Oleandomycin-Polymyxin-Sulphadiazine-Perfringens agar, one being prepared from a commercial, dehydrated product.

With skin samples taken from chicken carcasses at different stages of processing, the three direct plating media gave similar counts of *Cl. perfringens* whereas results obtained with the MPN method were consistently lower.

Although counts of *Cl. perfringens* from various further processed products were usually < 10/g, the three plating media showed similar specificity for this organism.

All media supported good growth of reference strains of *Clostridium perfringens* but it was found that physiologically similar species, including *Cl. absonum*, *Cl. paraperfringens* and *Cl. perenne* also grew uninhibited in these media and produced colonies identical with those of *Cl. perfringens*, thus indicating the need for confirmatory tests for *Cl. perfringens* when examining natural samples.

INTRODUCTION

Reports of the Public Health Laboratory Service for the years 1973–5 show that *Clostridium perfringens* continues to be the second most important cause of food poisoning in England and Wales (Vernon, 1977). The majority of outbreaks are attributed to red meat or poultry and involve problems of inadequate cooking, slow cooling and unrefrigerated storage, particularly in large-scale catering where meat is handled in bulk. Under these circumstances, the growth of *Cl. perfringens* in poultry meat can be very rapid (Mead, 1969).

Cl. perfringens is widely distributed in nature and occurs in the intestines of most warm-blooded animals including poultry (Smith, 1965; Smith, 1970); hence, it is to be expected that low numbers will occur on poultry carcasses whilst spores in particular may survive further processing (Mead & Impey, 1970; Gibbs, 1971; Lillard, 1971; Zottola & Busta, 1971; Hagberg *et al.* 1973).

Although many different media and methods have been developed for the selective isolation and enumeration of *Cl. perfringens* from faeces, food and water, little information is available on their specific application to the examination of

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poultry meat and poultry products. The purpose of the study was to evaluate certain of the media and isolation procedures which have been developed more recently.

MATERIALS AND METHODS

Reference organisms

The strains and their sources are given in Table 1. Cultures were maintained at room temperature in cooked meat medium (CMM) supplied by Southern Group Laboratory, Hither Green Hospital, London. For test purposes, fresh cultures were prepared in CMM and incubated at 37 °C for 24 h.

Table 1. *Reference organisms and their sources*

Species	Strains	Source
Cl. perfringens	2317	E. G. Harry; necrotic enteritis of chicken
	6.VO.2	Chicken caecum
	17.3.2*a*	Duodenum of hung pheasant
Cl. absonum	FT 230	G. Hobbs
	FT 231	
Cl. celatum	GD.1.B	A. H. W. Hauschild
Cl. paraperfringens	DS 4443	L. Ds. Smith
	DS 4446	
	DS 4624	
Cl. perenne	PH 2	Duodenum of hung pheasant
	PH 3	

Sampling of poultry meat and processed products

Chicken carcasses were sampled at three different stages during processing at a large, commercial processing plant: (i) after evisceration and spray-washing; (ii) after immersion chilling (iii) after freezing. At this processing plant, in-plant chlorination (20 parts/10^6) was being used for process water with additional chlorination of the chilling system to give a total available residual of *ca.* 50 parts/10^6.

Before freezing, carcasses were sampled at the processing plant whilst frozen carcasses were taken to the laboratory and thawed at 1 °C for 24 h before sampling. In each case the sample comprised 5–10 g of neck skin which was collected aseptically in a separate plastic bag. Samples taken at the processing plant were transported in ice to the laboratory.

Various further-processed products were purchased from local supermarkets Frozen items were thawed as before; these included the following chicken products: 'burgers', 'fingers', rissoles, 'sizzles' and sausages. Chilled, unfrozen products of chicken or turkey examined included cooked portions, sliced cooked meat and liver pâté.

Counting of Cl. perfringens

Samples were weighed and homogenized for 2 min in 20 ml of 0·1 % peptone diluent using a Colworth Stomacher 80 (Seward & Co. Ltd., Bury St Edmunds, Suffolk). Appropriate tenfold dilutions of the sample in 0·1 % peptone diluent

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were made just before inoculating the test media in a randomized sequence (Hauschild *et al.* 1977).

The media and methods studied were the following:

(1) Liquid enrichment involving the Differential Reinforced Clostridial Medium (DRCM) of Gibbs & Freame (1965) used for Most Probable Number (MPN) determinations with five replicates at each of four sample dilutions. The medium was boiled to expel oxygen and cooled before use. After incubation in air at 37 °C, cultures showing blackening of the medium at 24 or 48 h were streaked on the medium of Willis & Hobbs (1959), as used in the examination of poultry meat and processing plant samples by Gibbs (1973). In the present study, however, the Willis & Hobbs medium was used without the addition of milk (LEY).

(2) Direct plating using each of the following media: (*a*) Tryptose-Sulphite-Cycloserine agar without egg yolk (TSC minus EY) prepared according to Hauschild & Hilsheimer (1974*a*); this medium gave favourable results with meats and meat products when tested by the International Commission on Microbiological Specifications for Foods (ICMSF) as described by Hauschild *et al.* (1977); (*b*) Oleandomycin-Polymyxin-Sulphadiazine-Perfringens (OPSP) agar prepared according to Handford (1974) and shown by her to give higher counts of *Cl. perfringens* from foods with fewer false positives than several other media tested; (*c*) a dehydrated version of OPSP agar (Oxoid Limited, Basingstoke, Hants) prepared as described by the manufacturer from an agar base (CM543) to which were added supplements of the antibiotics (SR76) and sulphadiazine (SR77).

All agar plates were incubated at 37 °C for *ca.* 20 h in an anaerobic jar containing a mixture of hydrogen and 10%(v/v) carbon dioxide.

Tests used with all isolation media to confirm Cl. perfringens

With each plated sample, all black colonies growing at the highest dilution on the different test media were picked into CMM for overnight incubation at 37 °C. The resultant cultures were checked for purity by streaking on plates of nutrient agar containing 0·5% lactose and incubated anaerobically at 37 °C overnight. Colonies of presumptive *Cl. perfringens* were inoculated into lactose-gelatin and nitrate-motility media, the latter being supplemented with galactose and glycerol (Hauschild & Hilsheimer, 1974*a*). Liquefaction of gelatin was recorded up to 48 h. Nitrate reduction was determined according to Angelotti *et al.* (1962).

The same confirmatory tests were used for presumptive *Cl. perfringens* growing on LEY following the plating of blackened DRCM cultures.

RESULTS

Following reports that some strains of *Clostridium perfringens* are inhibited on OPSP medium (Handford, 1974; Hauschild & Hilsheimer, 1974*a*), preliminary tests were made with three reference strains isolated previously from chickens and pheasants. Each strain grew well and produced black colonies > 1 mm in diameter on each of the selective plating media whilst the higher recovery in DRCM (Table 2) may reflect the absence of selective inhibitors in this medium.

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Table 2. *Comparison of media for the counting of reference strains*

(Percentage recovery* using TSC minus EY as a standard.)

Species	Strain	OPSP	Oxoid OPSP	DRCM
Cl. perfringens	2317	104	63	190
	6.VO.2	103	64	433
	17.3.2a	77	37	158
Cl. absonum	FT 230	90	94	251
	FT 231	112	118	68
Cl. celatum	GD.1.B	NG	NG	NT
Cl. paraperfringens	DS 4443	91	90	141
	DS 4446	84	98	158
	DS 4624	96	92	84
Cl. perenne	PH 2	133	104	36
	PH 3	100	89	74

NG, no growth. NT, not tested.
* Mean of two trials.

Table 3. *Counting of* Clostridium perfringens *on poultry carcasses sampled at different stages of processing, comparing the DRCM method and three plating media*

		Confirmed count/g			
Processing stage	Sample no.	TSC minus EY	OPSP	Oxoid OPSP	DRCM
After spray-washing	1	450	360	420	86
	2	4000	4000	3800	85
	3	200	250	200	< 70
	4	540	890	830	< 70
	5	480	360	400	60
	6	810	820	1100	59
	7	1300	640	510	140
	8	1400	1000	1300	180
	9	2800	2400	2900	68
	10	300	370	320	< 50
	11	1200	1900	NT	260
	12	470	540	NT	250
After water chilling	1	1·5	4·5	1·5	0·6
	2	2·1	5·5	< 2·1	2·1
	3	< 1·9	5·7	< 1·9	0·8
	4	< 2·0	< 2·0	2·0	< 0·4
	5	1·9	< 1·9	1·9	< 0·4
	6	2·1	< 2·1	< 2·1	0·8
	7	22	20	14	2·8
	8	< 1·2	< 1·2	< 1·2	< 0·4
	9	1·9	< 1·9	< 1·9	< 0·4
	10	< 1·4	< 1·4	< 1·4	< 0·4
After freezing and thawing	1	7·8	2·0	NT	< 0·4
	2	1·0	< 1·0	NT	< 0·4
	3	2·0	3·0	NT	0·4
	4	2·9	2·0	NT	0·4
	5	7·5	3·2	NT	0·4
	6	3·0	6·0	NT	0·4
	7	4·0	9·6	NT	< 0·4
	8	< 1·0	1·0	NT	< 0·4

NT, not tested.

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On testing several other species of clostridia which are known to resemble *Cl. perfringens* in their physiological properties, it was found that all except *Cl. celatum* grew completely uninhibited in the selective plating media within 24 h and produced colonies identical in appearance to those of *Cl. perfringens*.

With raw poultry meat, the incidence of *Cl. perfringens* was highest on carcasses sampled immediately after evisceration and spray-washing whilst much lower counts were obtained after water chilling and subsequently after freezing and thawing (Table 3). Confirmed counts were similar for TSC minus EY and the two forms of OPSP; however, DRCM counts were consistently lower for samples taken at each of the three stages in processing, despite the fact that DRCM was used as a five-tube MPN method which permitted the testing of larger inocula than is practicable with direct plating. With carcasses sampled before chilling, the geometric mean counts for TSC minus EY, OPSP, OPSP (Oxoid) and DRCM were 796, 776, 768 and 112 respectively.

In the case of further-processed products sold frozen, *Cl. perfringens* was detected in low numbers (usually < 10/g) in 10/20 samples (Table 4). This total included one sample of rissoles which contained several hundred *Cl. perfringens*/g. Completely negative results were obtained from seven samples of products retailed under chill conditions, including cooked portions, sliced cooked meat and liver pâté.

Table 4. *Recovery of* Clostridium perfringens *from further-processed chicken products after thawing; DRCM method and three plating media compared*

		Confirmed count/g				
Sample type	No.	TSC minus EY	OPSP	Oxoid OPSP	DRCM	
Burgers	1	1·5	3·0	< 1·5	NT	NT
	2	< 1·8	< 1·8	< 1·8	NT	NT
	3	< 2·3	< 2·3	< 2·3	< 0·6	< 0·6
	4	2·7	< 2·7	< 2·7	< 0·6	< 0·6
Sausages	1	< 2·0	< 2·0	< 2·0	NT	NT
	2	2·1	6·3	< 2·1	NT	NT
	3	21	< 1·9	< 1·9	< 0·6	< 0·6
	4	< 2·5	2·5	2·5	< 0·6	< 0·6
Rissoles	1	940	690	560	NT	NT
	2	NT	180	92	NT	NT
	3	< 2·5	< 2·5	< 2·5	2·5	2·5
	4	< 2·7	< 2·7	< 2·7	3·0	< 0·6
Sizzles	1	< 1·8	< 1·8	< 1·8	NT	NT
	2	< 2·2	2·2	< 2·2	NT	NT
	3	16	5·8	< 2·7	0·7	< 0·6
	4	12	12	24	1·1	1·1
Fingers	1	< 1·8	< 1·8	< 1·8	NT	NT
	2	< 1·9	< 1·9	< 1·9	NT	NT
	3	< 2·2	< 2·2	< 2·2	< 0·6	< 0·6
	4	< 2·2	< 2·2	< 2·2	1·1	< 0·6

NT, not tested.

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On the plating media most samples of further-processed products in which *Cl. perfringens* was detected yielded colonies < 1 mm in diameter which may indicate a slower recovery of the organism following further processing and subsequent cold storage of the product. No single isolation medium appeared to be better than any other in yielding higher counts of *Cl. perfringens* but in most cases the numbers were too low to permit comparison.

Regardless of the type of sample, a high proportion of black colonies picked from the plating media were confirmed as *Cl. perfringens* (Table 5). However, a sausage

Table 5. *Confirmation of presumptive* Clostridium perfringens *from plating media*

Type of sample	Medium	No. of colonies tested	Percentage confirmed
Carcass during processing	TSC minus EY	109	98
	OPSP	100	88
	Oxoid OPSP	90	97
Frozen carcasses after thawing	TSC minus EY	14	100
	OPSP	12	100
	Oxoid OPSP	NT	—
Further processed products	TSC minus EY	35	77
	OPSP	30	90
	Oxoid OPSP	30	87

NT, not tested.

Table 6. *Presence of interfering organisms on selective plating media*

Sample no.	Colony count/g					
	TSC minus EY		OPSP		Oxoid OPSP	
	Cl. perfringens	Others	*Cl. perfringens*	Others	*Cl. perfringens*	Others
1	290	< 2	290	< 2	300	3300
2	160	< 2	190	1500	180	2400
3	210	< 2	200	2100	210	2600
4	200	< 2	200	1600	230	1300
5	120	< 2	160	1900	150	4100

sample yielded small black colonies of an unidentified, proteolytic clostridium on TSC minus EY but not on either OPSP medium and two samples of 'sizzles' contained strains of *Cl. perfringens* which were found subsequently to be lecithinase negative. These resembled strains associated previously with food poisoning outbreaks (Pinegar & Stringer, 1977) and were isolated on both TSC and OPSP media. An important difference between the plating media was in the incidence of interfering organisms. Examples of results obtained from carcasses sampled after spray washing are given in Table 6 and show that whilst the test media gave similar recoveries of *Cl. perfringens*, only TSC minus EY was free from interference by other organisms. With either of the OPSP media the large numbers present necessitated purification of strains picked from typical black colonies before sub-

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jecting them to confirmatory tests for *Cl. perfringens*. This is in accordance with previous findings for TSC and OPSP media when tested against a variety of foods (Hauschild & Hilsheimer, 1974*b*). In the case of DRCM, the number of blackened primary cultures usually exceeded the confirmed count of *Cl. perfringens* by ten to 100-fold.

DISCUSSION

TSC minus EY and OPSP prepared either from individual ingredients or a commercial dehydrated product gave similar results when used to count *Clostridium perfringens* in poultry meat and various further-processed items. The low incidence of the organisms in samples taken after chilling, freezing or further processing is consistent with evidence that chilling or freezing of poultry products reduces the viability of any *Cl. perfringens* present (Hagberg *et al.* 1973; Trakulchang & Kraft, 1977).

The DRCM method which involved non-selective enrichment followed by plating of blackened cultures on a differential medium, gave consistently lower counts than those obtained with the direct plating media. Probably this was due to interference from competing organisms since *Cl. perfringens* was invariably outnumbered by other organisms which also produced blackening in DRCM. There appears to be a need for an efficient selective enrichment medium to facilitate the isolation and counting of *Cl. perfringens* from foods and for this purpose the milk medium developed by Erickson & Deibel (1978) would merit consideration.

Unlike the ICMSF study (Hauschild *et al.* 1977) the test samples were not pre-incubated at a temperature likely to encourage growth of *Cl. perfringens* in order to increase the numbers of the organism and thus facilitate the comparison of isolation media. Pre-incubation of samples changes the physiological state of the organisms being sought and alters the relative proportions of interfering types, factors which may influence the performance of the selective media being studied. No attempt was made in the present study to distinguish between vegetative cells and spores or to activate spores by heat treatment because Zottola & Busta (1971) obtained fewer positive samples when heating was used to determine the incidence of *Cl. perfringens* spores in various turkey products.

For the purposes of routine quality control in the poultry industry it may be sufficient to determine counts of 'presumptive' *Cl. perfringens* on either TSC minus EY or OPSP without proceeding to the relatively laborious confirmatory tests. Where more precise information is needed, however, it would be necessary to subject cultures to further tests since strains of species resembling *Cl. perfringens* in their physiological properties were found to be indistinguishable from this organism on TSC or OPSP media. Of the species tested, both *Cl. paraperfringens* and *Cl. perenne* have been isolated previously from chickens or pheasants (Barnes *et al.* 1978). Should confirmation be required, the use of TSC minus EY is to be preferred because colonies can be picked directly whereas those from OPSP media have to be purified.

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