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Antibiotic residues in meat in the United Kingdom; an assessment of specific tests to detect and identify antibiotic residues

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SUMMARY

Investigations were conducted between 1977 and 1979 to assess the performance of microbiological tests for detecting and identifying residues of therapeutic-type antibacterial substances in meat and offal. Of the 5442 home-produced meat samples examined, 34 (0·63%) showed inhibitory activity in the screening test, which used *Bacillus subtilis* BGA and *Micrococcus luteus* as indicator organisms. Identification by electrophoretic and thin-layer chromatography/bio-autography techniques confirmed that only two of the 34 screen failures were due to true antibacterial residues: a pig sample contained a trace of penicillin and a horse sample contained a trace of an incompletely identified substance resembling a tetracycline. Twelve of the other 32 failures in the screen test were due to naturally produced inhibition and were, thus, falsely positive, whilst the remainder were shown to be negative. All of the 85 (8·7%) screen test failures from the 972 imported meat and offal samples tested were falsely positive. Additional samples from certain animals known to have been given antibiotic treatment were tested concurrently to give a more searching indication of screen and identification test efficacy.

INTRODUCTION

There is mounting evidence to indicate that antibiotic residues in food could be a factor in the emergence of antibiotic-resistant bacteria, especially salmonellae, in human and animal infections (Anderson & Smith, 1972; Anon. 1974*a*; Anderson, 1975; Threlfall, Ward & Rowe, 1978*a*, *b*). In developed countries it is universally recognized that measures are needed to restrict the potential problems resulting

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from the use of antibiotics in animal husbandry, whether as prophylactic or therapeutic agents or as growth promotors in feeding stuffs (Report, 1969).

In the United Kingdom, the Medicines Act 1968 places controls upon the supply and use of antibiotics and other antibacterial agents by means of a licensing system. The control of residues as such has, in practice, been confined to the dairy industry especially with respect to antibiotic residues (usually penicillin) in milk, either by means of the Food and Drugs Act 1955 or through penalty schemes operated by the milk marketing boards. Several countries have introduced specific legislation requiring meat samples to be tested for the absence of antibiotic residues. For example, in the Federal Republic of Germany the carcasses of 1 % of beef cattle and 2 % of veal calves must be tested by a prescribed method (Anon. 1974*b*). The Third Country Directive of the EEC (European Communities Council Directive, 1972) requires that meat imported into the Community shall be free from antibiotic residues and it is expected that a forthcoming directive on intra-Community trade in meat will contain a similar provision.

Published information concerning antibiotic residues in meat in the United Kingdom has been limited as far as we are aware to data on certain offals mentioned by Smither (1978). During the period 1977 to 1979 further investigations were conducted, in which meat produced in the United Kingdom was initially tested at the Veterinary Investigation Centre, Reading (VICR), whilst meat and offal imported into the United Kingdom was tested at the Laboratory of the Government Chemist, London (LGC). Screening for antibacterial activity was by a modification of the Four Plate Test (FPT) discussed by Nouws, Van Schothorst & Ziv (1979), which itself was derived from the German 'Hemmstofftest' (Anon. 1974*b*). The modified procedure described in this paper was developed over several years during studies by an EEC Expert Committee. Throughout the testing period the efficacy both of the screening procedure and of a high voltage electrophoretic technique to identify antibiotics (Smither & Vaughan, 1978) were evaluated by examination of additional samples derived from casualty and *post mortem* animals received at VICR, some of which had a known history of recent antibiotic treatment.

MATERIALS AND METHODS

Sampling

UK-produced meat

A total of 5442 fresh meat samples were taken hygienically by staff of the Ministry of Agriculture, Fisheries and Food Territorial Divisional Veterinary Office from 26 nationally distributed export abattoirs in ten regions. To avoid spoiling the appearance of carcasses and thus reducing their value, samples (approximately 150–200 g) from cattle, pigs and horses consisted mainly of diaphragm muscle, whilst neck muscle was considered to be more suitable for lamb. Samples were transferred to resealable polythene bags, quickly deep-frozen, packed into polystyrene boxes containing two Freezella bags (Insulex Ltd) and dispatched to VICR. Arrival within 12 h of freezing ensured that the samples remained frozen and suitable for testing.

Table 1. *Types of imported meat and offal samples tested*

	Type of produce				
	Pig	Sheep	Beef	Veal	Goat
Offal	Kidney Liver	Kidney Liver Heart Sweetbreads	Kidney Liver Heart	Kidney	
Muscle	No pig muscle tested	Chop Chump chop Loin chop Neck Neck chop Neck joint Neck ½-shoulder Leg Loin Racks Trunk	Beef Beef ribeye Crop Flank Fillet Hind Loin Rump Striploin T-bone Tenderloin Thick skirt Topside	Veal Thick veal Skirt Thick skirt Flank ¼-flank Cutlet Best end Leg Loin	Neck

Imported meat

A total of 972 deep-frozen meat and offal samples from nine countries arriving at six major sampling centres, including five ports and another source, were taken hygienically from coldstores or meat inspection facilities by Port Health Authority officers or City of London Meat Inspectorate officers. Where possible, pre-cut samples of meat such as chops were taken, but where whole carcasses only could be sampled, portions such as lamb neck were taken using hand or band-saws. Alternatively, samples were taken from frozen blocks of meat using sterile knives although exceptionally, from one centre, 'off-cut' samples had to be accepted. The various cuts are listed in Table 1. Samples (approximately 150–200 g) were transferred to labelled, resealable polythene bags and within a short time were delivered, chilled, directly to LGC (local centre samples) or placed in a deep-freeze cabinet (port samples). The latter were maintained in a deep-frozen state during transit to LGC by packing with Freezella bags in polystyrene boxes and total transportation time never exceeded 6 h.

Immediately upon arrival at the respective laboratories samples were inspected for quality and placed in a deep-freeze (−20 °C) prior to testing.

The Four Plate Test (FPT) for antibiotic screening

Organisms

The test organisms, *Micrococcus luteus* NCTC 8340 (ATCC 9341) and *Bacillus subtilis* BGA (Bundesgesundheitsamt, Berlin) were maintained on slopes of Standard II Nähragar (Merck, Cat. No. 7883) containing additional KH_2PO_4 (1 g/l), final pH 7·0, incubation being at 30 °C for 16–18 h. A suspension of *M. luteus* was prepared freshly each day by inoculating Brain Heart Infusion

broth (Oxoid) and incubating at 37 °C for 24 h. Spores of *B. subtilis* were obtained by inoculating a suspension of vegetative cells into a Roux bottle containing 250 ml of Standard II Nähragar, pH 7·0, and incubating at 30 °C for 10 days. They were harvested with sterile 0·8 % (w/v) sodium chloride solution and centrifuged (3000 *g* for 10 min). Washing was repeated, when the cells were finally resuspended in saline and heat treated (70 °C for 30 min). The concentration of spores was adjusted to about 10^7/ml, this suspension being stable for several months if stored at 4 °C.

Assay plates

Volumes (100 ml) of Standard II Nähragar were adjusted with 0·1 N-HCl or 0·1 N-NaOH at 48–50 °C, so that at 25 °C they were pH 6·0, pH 7·2 and pH 8·0 (for *B. subtilis*) and pH 8·0 (for *M. luteus*). To enhance the detection of sulphonamides, trimethoprim, to a final concentration of 0·05 μg/ml of agar, was added to the *B. subtilis* pH 7·2 agar. The organisms were added to the agar to give a colony count of the order of 10^4/ml. The seeded agar was poured into levelled sterile disposable polystyrene assay plates, 23 cm square and 1·8 cm deep (Gibco Bio-cult Ltd, Nunc Cat. No. N-1015) to give a layer 2 mm thick. Prepared plates were either used immediately or could be stored for a maximum period of 4 days at 4 °C.

Preparation of samples

Samples were removed from the deep-freeze and allowed to reach a temperature of about −5 °C, before the outer (contaminated) surface was removed with a sterile scalpel. A cylindrical piece of meat was removed from each sample using a sterile cork borer (8 mm internal diameter), and 8 fat-free discs, 2 mm thick, were cut from it. Two discs were placed in diagonally opposite positions on each of the four seeded test plates using sterile forceps. Seventeen samples (34 meat discs) plus two antibiotic control discs were accommodated on a single plate in a 6 × 6 arrangement. Utensils were scrupulously cleaned and re-sterilized between samples to avoid contamination. The *B. subtilis* plates were incubated at 30 °C for 18–24 h whilst the *M. luteus* plate was incubated at 37 °C for 18–24 h. A positive test result was recorded when both meat discs on any plate gave a continuous annular zone of inhibition of not less than 2 mm across. Samples giving equivocal results were always repeated and if again the results were doubtful they were considered as being negative.

Control antibiotic discs

The sensitivity of all test plates was monitored by applying 6 mm diameter filter paper discs impregnated with sodium benzylpenicillin (0·01 i.u./disc), streptomycin (0·5 μg/disc) or sulphadimidine (0·5 μg/disc) (Mast Laboratories, Liverpool) which gave annular zones of inhibition of a specified minimum size, that is ⩾ 6–8 mm extending from the discs.

Table 2. *Detection of inhibitory substances in meat and offal samples*

Type of produce	'Four plate test' result			
	UK-produced meat		Imported meat	
	Pass	Fail	Pass	Fail
Beef	2072	4	88 (1)*	12
Veal	0	0	94 (2)	12
Sheep	1242	2	525 (8)	0
Goat	0	0	6	0
Pig	1835	27	0	0
Horse	259	1	0	0
Heart	0	0	64 (14)	0
Kidney	0	0	86 (20)	8
Liver	0	0	13 (13)	53
Sweetbreads	0	0	11 (5)	0
Totals	5408	34	887 (63)	85

* Figures in brackets represent number of samples in which zones of inhibition <2 mm wide occurred in one or more plates.

Electrophoresis and t.l.c./bio-autography

Positive FPT samples from both UK-produced and imported meat were examined further at LGC by high voltage electrophoresis (Smither & Vaughan, 1978) to identify antibiotics, and t.l.c./bio-autography (Smither, 1978) to establish the presence of naturally produced inhibitors. The exudates obtained from thawed tissues as well as extracts from freeze-dried material were used for electrophoresis. In addition, 70 samples from 31 *post mortem* animals, 34 samples from 18 casualty (emergency-slaughtered) animals and 21 turkey sera, all originating from VICR and failing the FPT during routine analysis, were tested at LGC for antibiotic identity.

RESULTS

UK-produced meat

Thirty-four (0·63 %) of the 5442 UK-produced meat samples (Table 2) failed the FPT on initial screening, 27 (79·4 %) of the failures being derived from pig meat. On retesting the FPT failures at LGC, electrophoresis and t.l.c./bio-autography revealed that 20 failures were actually negative and that 12 other failures were caused by naturally produced inhibitors similar to or identical to those described by Smither (1978), and could therefore be considered as falsely positive with respect to the test. Only two failures in the FPT were caused by the presence of antibiotics. One pig sample contained a trace of penicillin and a horse sample contained a trace of an incompletely identified antibacterial substance which resembled a tetracycline both in its migration behaviour during electrophoresis and in its response to a tetracycline-resistant strain of *Bacillus cereus*.

Imported meat

Eighty-five (8·7 %) of the 972 imported meat and offal samples (Table 2) failed the FPT, of which 76 (89·4 %) originated from one sampling centre. Thus, all beef (12), veal (12) and kidney (8), as well as 44 of 53 liver FPT failures were accounted for by samples from this source. The most striking result was that for liver, where every sample (including 13 that passed the FPT) produced inhibitory activity. Many of the liver samples failing the FPT produced annular zones of inhibition in excess of 6 mm (which, incidentally, corresponded in diameter to the zones obtained for the standard antibiotic discs). Electrophoresis and t.l.c./bio-autography confirmed that all FPT failures were due to naturally produced inhibitors.

Throughout this study the most sensitive plate for detecting antibacterial substances was the *B. subtilis* (pH 6·0) plate, on which about 80 % of failures, either with this plate alone or in combination with other plates, occurred.

False positive results

False positive results occurred in response to various microbial processes. Firstly, meat discs applied to test plates could already contain natural inhibitors which were presumably derived from earlier bacterial activity within the original sample (Smither, 1978). In support of this theory it was found that these samples were invariably heavily contaminated with bacteria, and that extracts of freeze-dried sample material were inhibitory to test bacteria. Secondly, contaminating organisms present on the meat discs could produce inhibition *in situ* on assay plates during incubation. In this case, inhibitors appeared to develop either directly from the decomposition of meat by contaminating organisms or as an antagonistic response between the contaminants and the assay organism, but generally only in the presence of certain unknown factors derived from the sample. In contrast to the first process above, extracts of freeze-dried sample material were not inhibitory to test bacteria. It is likely that both processes could occur simultaneously. The production of direct 'antibiotic' activity from the contact of certain contaminants and the assay organisms has been demonstrated *in vitro* in the absence of sample tissue juices, but no attempt was made to identify the substances responsible for such activity.

Organisms involved in inhibitor production included *Bacillus cereus*, *Bacillus licheniformis*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Lactobacillus* sp. Strongly lipolytic organisms that have been identified and which may be indirectly involved in inhibitor production include *Serratia liquefaciens* (*Enterobacter liquefaciens*), *Pseudomonas putida*, *Candida lipolytica* var. *deformans* and *Candida zeylanoides*.

Additional samples

It was difficult to judge the efficacy of the FPT by reference only to the home-produced and imported samples, since the vast majority of material tested had given negative results. Accordingly a more detailed study was made of the two additional series of samples which had failed the FPT.

Table 3. *Identification of antibacterial substances in* post mortem *animal samples failing the FPT*

Animal	Age	Treatment*	Electrophoresis/t.l.c. result* Kidney	Liver	Muscle
Cattle	u†	4d, Tb, 1 injection C	—	—	Ot‡
	5w§	Tb, Sdm, F, A	Sdm	Sdm	Sdm
	2w	C	ni	ni	ni
	3d§	u†	ni	—	−ve
	12d	Ot	Ot	—	Ot
	21d	C	ni	—	−ve
	2–3w	u	P	—	P
	37d	Last 4d Sdm, C Ot	Sdm	Sdm	Sdm
	1w	2 doses Tb before death	Tr, Sdz	Tr, Sdz	Tr, Sdz
	1·5d	A, P, S day before death	C‡, S	C‡, S	C‡, S
	1–2w	T(?)Tr	Ot, Tr	—	Ot, Tr
	Few w	Probably A	ni	ni	C‡
	u	T, Tr	ua‡	—	—
	u	Probably A	—	—	C‡
	u	Tb	Tr, Sdz	Tr, Sdz	Tr, Sdz
	u	Ot	Tr‡	ni	—
	u	Ot	—	—	Ot, ua‡, ni
	u	Ot	Ot, ua‡	Ot, ua‡	Ot, ua‡
	u	Tb, A	Tr, Sdz, A, ua‡	Tr, Sdz, A, ua‡	Tr, Sdz, A, ua‡
	u	Tb, A	Tr, Sdz, A, ua‡	Tr, Sdz, A, ua‡	Tr, Sdz, A, ua‡
Pig	5m§	P, S in last 2d	P	ni	−ve
	u	P	—	—	P
	u	200g P, 150g Sdm ton^{-1} feed	ni	—	−ve
	u	10ml PP+DS day before death	P, DS	—	P, DS
	u	80g PP, 160g Ct, 160g Sdm ton^{-1} feed	−ve	—	−ve
	u	80g PP, 160g Ct, 160g Sdm ton^{-1} feed	−ve	—	−ve
	u	Ot in feed	ni, ua†	—	ni, ua‡
	u	Ot in feed (+medication?)	P‡	ni	P‡
Sheep	1y§	P, T, Ot	P, Ot	P, Ot	P
	Mature	u	P	—	P
Giraffe	6d	u	ni	—	ni

* A, ampicillin; C, chloramphenicol; Ct, chlortetracycline; DS, dihydrostreptomycin; F, framycetin; Ot, oxytetracycline; P, penicillin; PP, procaine penicillin; S, streptomycin; Sdm, sulphadimidine; Sdz, sulphadiazine; T, tetracycline; Tb, tribrissen (trimethoprim+sulphadiazine); Tr, trimethoprim; ni, naturally produced inhibitor causing false positive result; ua, unidentified antibacterial; −ve, inhibition not confirmed; —, no sample available.

† u, unknown age or treatment.

‡ Confirmed antibiotic does not correspond to stated treatment.

§ d, day; w, week; m, month; y, year.

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Table 4. *Identification of antibacterial substances in casualty animal samples failing the FPT (treatment, if any, unknown)*

	Electrophoresis/t.l.c. result*		
Animal	Kidney	Liver	Muscle
Cattle	ni	ni	—
	ni	P	ni
	P	—	−ve
	ni	—	−ve
	ni	—	—
	ua†	—	−ve
	—	—	ni
	DS	DS	—
	ni	—	—
	DS	DS	—
	S	ni	—
	ni	P	—
	−ve	−ve	—
Pig	ni	—	ni
	ni	—	ni
	ni	—	−ve
	ni	ni	−ve
	DS	—	—

* DS, dihydrostreptomycin; P, penicillin; S, streptomycin; ni, naturally produced inhibitor causing false positive result; −ve, inhibition not confirmed; —, no sample available.

† Unidentified antibacterial, sample gave zone of inhibition in FPT assay procedure, but no zone with electrophoresis.

Veterinary investigation-samples

From a series of 70 *post mortem* samples (Table 3), 46 (from 22 animals) were found to contain antibiotic residues identifiable by electrophoresis. In total, nine different antibacterial substances were positively identified. Twenty-two samples (from 12 animals) contained substances different from the supposed treatment and, of these, 13 (from six animals) contained unidentified antibacterial substances. The unidentified substance in samples from the last three cattle were apparently identical. In samples from three other cattle, chloramphenicol was found where the supposed treatment was ampicillin. In five instances where named antibiotics had been administered to pigs via their feed, detectable residues were not found. Similarly, in cases where multi-component treatment was given, detectable residues were not invariably found, and therefore each individual antibiotic substance could not always be identified by electrophoresis. Where streptomycin or dihydrostreptomycin had been administered, identification was achieved using tissue exudates in preference to extracts of freeze-dried preparations. Naturally produced inhibition accounted for FPT failures in 13 samples from nine animals.

Of the 34 casualty samples examined (Table 4), nine (from seven animals) contained antibiotic residues which could be identified by electrophoresis. In addition, sulphaquinoxaline was identified in 3 of 21 samples of turkey serum examined.

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DISCUSSION

Based on data from samples of animals known to have been given therapeutic doses of antibiotics, it was clear that the FPT was sensitive enough to detect residues of many antibiotics in meat and offal. Without the data from these additional samples, however, it would have been difficult to draw conclusions about the performance of the FPT, since the majority of results were negative. By interpolation it was concluded that the present studies indicated an encouraging situation where currently there appears to be little problem concerning antibiotic residues in home-produced meat (only 2 of 5442 samples contained detectable levels of therapeutic antibiotics) and no problem with imported meat.

A word of caution should be expressed regarding the imported meat samples provided by one sampling centre, of which 76 (46·6%) of 163 samples failed the FPT. This was consistent with our initial observations on receipt, that samples originating from this centre were generally of poor quality. It is believed that the results do not reflect the true quality of the vast bulk of meat handled there but simply relate to the unacceptable samples furnished to the collecting officers. Since the purpose and scope of the FPT is the detection of residues of antibacterial substances in fresh lean meat it should be reiterated that samples of stale, contaminated meat will give fallacious results. In this context we have no reason to doubt that fresh offal can be tested adequately by the FPT, although we are well aware that these tissues notoriously cause false positive results, probably because of their higher susceptibility to microbial attack.

An important feature of the FPT was the inclusion of a plate to detect sulphonamide compounds which, especially in combination preparations containing trimethoprim, are an increasingly strategic part of the veterinarian's drug armoury. It was unfortunate that the use of trimethoprim to enhance the detection of sulphonamides (and, fortuitously, certain other antibiotics and some naturally produced inhibitors) as shown during ancillary studies, marginally reduced the sensitivity of *B. subtilis* BGA to trimethoprim itself. The current trimethoprim plate is superior, however, for detecting sulphonamides compared to its predecessor, which used a strain of *Escherichia coli* in a chemically defined medium at pH 7·2. Both sensitivity and clarity of zones of inhibition have been improved.

The major drawback of a test like the FPT is that it is only capable of detecting inhibitory activity. It cannot discriminate amongst antibiotics, other drugs with inhibitory activity or naturally produced inhibitors, although it can provide limited information through the response of the two assay organisms to inhibitors at different pH levels. In certain continental countries the present practice is to pass or fail carcasses for human consumption on the findings of the FPT or a similar test with no follow-up confirmatory procedure, and it is conceivable that erroneous interpretation of FPT results might lead to unnecessary economic loss. Since some false positive results may be produced *in situ* due to growth of contaminating organisms it is imperative, when applying a test of this nature, to have the supplementary facility for identifying bacteriologically active substances, and to be able to distinguish antibiotic activity from naturally produced inhibition.

Some unusual results emerged from the *post mortem* animal samples, in particular, the observation that chloramphenicol was identified in three apparently unrelated animals said to have been treated with ampicillin. This confirms the difficulty of obtaining an accurate record of treatment from the animal owner who is often unaware of the precise treatment that has been administered to his stock. The detection of chloramphenicol in samples by the FPT is not in accord with the observations of Nouws *et al.* (1979) who maintained that the FPT method was incapable of detecting this drug. Indeed, our own observations (unpublished data, 1978) from rabbits experimentally injected with therapeutic doses of chloramphenicol would support their contention. It is possible that doses exceeding normal recommended levels may have been used or that the rate of uptake and retention may be markedly different in ill or diseased animals (Baggot, 1977).

Another feature of this test about which some doubt exists concerns the retention of the *B. subtilis* (pH 8·0) plate. Nouws *et al.* (1979) regarded this plate as superfluous since the *M. luteus* (pH 8·0) plate afforded better detection of macrolide antibiotics. From our own experience we would generally concur, although where low levels of aminoglycoside compounds are present the *B. subtilis* (pH 8·0) plate is still necessary for detection.

The inability of the electrophoresis technique to identify all the antibacterial substances found in *post mortem* samples may be accounted for by (1) antibiotics not previously encountered, (2) the presence of drugs such as antiprotozoals which can have inhibitory properties, (3) components of combination therapy intended for purposes other than the direct control of infection (for example certain steroids can be inhibitory), (4) sample contamination with farm or *post mortem* room disinfectants or antiseptics. In the instances where not all antibiotics in combintion or multicomponent therapy were identified, it is probable that the substances concerned had been either eliminated completely from the animal body or metabolized to low, undetectable levels or inactive derivatives. In the case of streptomycin and dihydrostreptomycin it was found particularly useful to apply tissue exudates directly to the electrophoresis plate instead of extracts from freeze-dried material, because of poor recovery from the latter. Low recovery was attributed to poor antibiotic solubility in the extraction solvent, acetonitrile-distilled water (9:1, by volume).

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