J. Hyg., Camb. (1981), **86**, 129
Printed in Great Britain

The occurrence and significance to animal health of *Leptospira, Mycobacterium, Escherichia coli, Brucella abortus* and *Bacillus anthracis* in sewage and sewage sludges

By P. W. JONES, LYNNE M. RENNISON, P. R. J. MATTHEWS, P. COLLINS and ANNE BROWN

A.R.C. Institute for Research on Animal Diseases, Compton, Newbury, Berkshire

(*Received* 8 *April* 1980)

SUMMARY

Samples of sewage, sewage sludge and sewage effluent from one or more of four sewage treatment plants were examined for the presence of *Leptospira, Mycobacterium, Escherichia coli, Brucella abortus* and *Bacillus anthracis*. *Brucella abortus* and *Bacillus anthracis* were not isolated. Eleven strains of *E. coli* potentially enteropathogenic for calves or piglets, eight pathogenic strains of *Mycobacterium* and one pathogenic *Leptospira* strain were isolated from 101, 189 and 189 samples respectively.

Sewage sludge is not considered to play a major part in the epidemiology of disease caused by these organisms.

INTRODUCTION

Sewage sludge may be a valuable source of fertilizer when disposed of on agricultural land. However, it may contain organisms potentially pathogenic for grazing cattle. Jones *et al.* (1980) reported on the isolation of salmonellas from samples of sewage, sewage sludge and sewage effluent from eight sewage treatment plants within the Thames Water Authority. They considered salmonellas to constitute the most important hazard to grazing animals but a lesser hazard might be presented by organisms such as *Brucella abortus, Bacillus anthracis*, enteropathogenic strains of *Escherichia coli*, pathogenic mycobacteria and pathogenic leptospires.

This communication describes the attempted isolation of these organisms from four of the sewage treatment plants studied by Jones *et al.* (1980).

MATERIALS AND METHODS

Sewage treatment plants

The sewage treatment plants from which samples of sewage were examined have been described previously (Jones *et al.* 1980). Samples from one plant (plant C) which received effluent from two tanneries were examined for *Bacillus anthracis*.

0022-1724/81/0031-1980 $01.00

Samples from plant D which received effluent from a cattle market were examined for *Escherichia coli*, *Brucella abortus*, pathogenic mycobacteria and pathogenic leptospires; samples from plant F, a large urban sewage treatment plant, were examined for pathogenic leptospires and pathogenic mycobacteria.

Isolation and enumeration of coliforms and Escherichia coli

The concentration of coliforms in samples from plant D was determined by spreading 0·1 ml volumes of appropriate dilutions of sample in 0·85 % (w/v) saline over the surface of MacConkey agar plates; the plates were incubated at 37° C for 24 h and lactose-fermenting colonies with typical coliform morphology were counted. A selection of such colonies was removed from each plate for identification. *Escherichia coli* was also isolated by spreading 0·05 ml volumes of sample on two blood agar plates (bovine blood, 7·5 %). An antibiotic multodisc (Oxoid 30-3G) was placed on the surface of one of the plates and both were incubated at 37 °C for 18 h. Colonies resembling *E. coli* in colony morphology, and particularly those surrounded by a zone of haemolysis, were removed for identification. Isolates were identified as *E. coli* according to the method of Buchanan & Gibbons (1974) and serotypes commonly associated with disease in calves and pigs were identified serologically according to the method of Sojka (1965).

Isolation of Brucella abortus

Samples from plant D were inoculated heavily on three replicates of albimi agar (Joint F.A.O./W.H.O. Expert Committee on Brucellosis, 1958) and incubated at 37 °C for up to seven days in an atmosphere containing 10 % carbon dioxide. Colonies resembling those of *Brucella abortus* morphologically were identified biochemically according to Buchanan & Gibbons (1974) and examined for agglutination in polyvalent *Brucella abortus–Brucella melitensis* antiserum.

Isolation of Bacillus anthracis

Twenty-five ml of each sample from plant C was mixed with 75 ml of distilled water, stored at 4 °C and shaken several times over a period of 18 h. The supernatant was removed and heated at 70 °C for 10 min and, after cooling, inoculated onto two replicates each of Pearce and Powell agar (Pearce & Powell, 1951) and propamidine agar (Morris, 1955). The supernatant was then centrifuged at 800 ***g*** for 30 min and the deposit was also used to inoculate Pearce and Powell and propamidine agar (as above). One replicate of each medium was incubated at 37 °C and the other at 40 °C for 48 h. Colonies resembling *B. anthracis* in colony morphology were inoculated into bacto-tryptose broth and incubated at 37 °C for 18 h. The broth was diluted 1/100 in 0·85 % (w/v) saline and 0·1 ml inoculated intraperitoneally into two mice (adult male, Compton white). The deposit produced by centrifugation was also inoculated subcutaneously into two guinea-pigs and four mice as follows: one guinea-pig 5 ml, one guinea-pig 3 ml, two mice 1 ml, two mice 0·5 ml. The guinea-pigs were immunized three days prior to inoculation by intramuscular injection of 0·5 ml of antiserum prepared against *Clostridium*

perfringens, *C. septicum*, *C. oedematiens* and *C. tetani*. All animals were examined daily for up to 14 days and post-mortem examinations carried out on animals which died. At autopsy, heart blood, spleen and material from the site of inoculation of each animal was inoculated onto blood agar and incubated at 37 °C for 24 h. Colonies resembling *B. anthracis* were identified according to the method of Buchanan & Gibbons (1974) and by mouse pathogenicity as described above. Smears prepared from heart blood, spleen and site of inoculation were stained by Giemsa's method and examined as an aid to identification.

Isolation of mycobacteria

Mycobacteria were isolated in Stuart's basal medium (Stuart, 1965) containing 10 ml egg yolk emulsion (Oxoid SR47), 4 ml 9% bovine albumin (Armour Laboratories), (Benzylpenicillin BP, Glaxo Laboratories), penicillin (100 units/ml), and amphotericin B (50 μg/ml) (Fungizone, E. R. Squibb & Sons Inc., New York) in 96 ml of base. Ten ml of sample was centrifuged at 800 *g* for 15 min and 1 g of deposit was added to 10 ml of 0·26% benzalkonium chloride in 10% trisodium phosphate. The resultant suspension was filtered through muslin, incubated at 37 °C for 30 min and re-centrifuged at 800 *g* for 15 min. After centrifugation the deposit was resuspended in 2 ml 0·85% (w/v) saline containing amphotericin B (1000 μg/ml) and neutralized with 1 N hydrochloric acid. The suspension was distributed on five slopes of isolation medium (as above) and five slopes of isolation medium with the addition of 3·0 μg/ml crude mycobactin (Stuart, 1965). The slopes were incubated at 37 °C and examined at intervals for up to six months. Colonies morphologically resembling mycobacteria were identified biochemically and serologically as described by Matthews, Collins & Jones (1976).

Isolation of leptospires

Samples of final effluent were reduced from an initial volume of 20 ml to 5 ml by centrifugation at 800 *g* for 15 min. Depending upon their consistency, 1 ml or 1 g of other samples was mixed with 5 ml of phosphate-buffered saline and filtered through sterile muslin. Suspensions were then centrifuged at 800 *g* for 30 s and 5 ml of supernatant was filtered through a 0·45 μm membrane (Millipore) in a Hemming filter centrifuged at 800 *g* for 15 min. Drops of 0·02 ml of the undiluted filtrate or the filtrate diluted 1/10, 1/100 or 1/1000 in 0·85% (w/v) saline were inoculated in two replicates each of three media. These were Ellinghausen's medium (Ellinghausen & McCullough, 1965) containing either rabbit serum (1%) and agar (1·5%) or rabbit serum (1%), 5-fluorouracil (100 μg/ml) (Johnson & Rogers, 1964*a*), amphotericin B (50 μg/ml) (Jones & Matthews, 1975) and agar (1·5%) or rabbit serum (1%), sulphathiazole (50 mg/litre), neomycin sulphate (5 mg/litre), actidione (0·5 mg/litre) (Cousineau & McKiel, 1961) and agar (1·5%).

The media were incubated at 29 °C and growth of leptospires monitored by dark-field microscopy at two days, five days and then at weekly intervals up to six weeks. All isolates were inoculated into 8-azoguanine medium (Johnson & Rogers, 1964*b*) and submitted to the Leptospirosis Reference Laboratory, Colindale, for serological typing.

Table 1. *Concentration of coliforms (total coliform count) in samples from treatment plant D*

Sample	Range/100 ml	Geometric mean/100 ml
Settled sewage	$6{\cdot}3 \times 10^4$–$8{\cdot}5 \times 10^6$	$3{\cdot}6 \times 10^5$
Raw sludge	$1{\cdot}0 \times 10^5$–$4{\cdot}7 \times 10^9$	$1{\cdot}7 \times 10^7$
Centrifuged sludge cake	$4{\cdot}5 \times 10^5$–$6{\cdot}6 \times 10^8$	$6{\cdot}3 \times 10^6$
Drying bed sludge	<10–$6{\cdot}1 \times 10^7$	$9{\cdot}2 \times 10^4$
Final effluent	<10–$7{\cdot}0 \times 10^4$	$7{\cdot}9 \times 10^2$

pH and total solids content of samples

Where possible, pH was measured on the undiluted sample using a membrane electrode. Total solids were determined by heating 10 g of each sample to constant weight in a hot-air oven at 104 °C. The residual solids were weighed and recorded as a percentage of the original weight of the wet sample.

RESULTS

Isolation and enumeration of coliforms and Escherichia coli

All samples (101) from plant D examined by the surface spread count method contained coliforms in concentrations shown in Table 1. A reduction in concentration of coliforms from settled sewage to final effluent of 99·8 % and from raw sludge to drying bed sludge of 99·9 % was observed.

A total of 125 isolates were identified as *E. coli* of which 23 were haemolytic on blood agar. Eleven were identified as belonging to 'O' groups frequently involved in disease in calves and piglets.

Examination for Brucella abortus

Brucella abortus was not detected in 101 samples from plant D.

Examination for Bacillus anthracis

Ninety samples from plant C, five samples of tannery effluent and six samples of soil from a field over which final effluent from plant C had been spread were examined. *Bacillus anthracis* was not isolated. Two strains of *Bacillus* which resembled *B. anthracis* morphologically and which were pathogenic for mice in a dilution of 1/100 of an 18 h broth were isolated at post-mortem from two guinea pigs which had been inoculated with a sample of raw sludge.

Isolation of leptospires

Thirty strains of *Leptospira* were isolated from treatment plants D (101 samples) and F (88 samples) (Table 2). Twenty-nine grew in 8-azoguanine broth, were agglutinated by *Leptospira biflexa* antiserum and were confirmed as saprophytic strains by the Leptospirosis Reference Laboratory. One strain which failed to grow in 8-azoguanine was provisionally identified as a new member of the *Leptospira tarassovi* serotype.

Table 2. *Isolation of leptospires from two sewage treatment plants*

Treatment plant	Sample	No. examined	Leptospires isolated
D	Settled sewage	23	1
	Raw sludge	23	0
	Processed sludge	23	3
	Final effluent	32	11
F	Settled sewage	22	3 (1 pathogenic)
	Raw sludge	22	0
	Anaerobically digested sludge	22	1
	Final effluent	22	11

Table 3. *Isolation of mycobacteria from treatment plant D*

		Number and identity of mycobacteria isolated	
Sample	No. examined	Fast-growers	Slow-growers
Settled sewage	23	1 *M. fortuitum* (1)	1 *M. paratuberculosis* (1)
Raw sludge	23	13 *M. fortuitum* (6) *M. peregrinum* (6) *M. gordonae* (1)	4 *M. scrofulaceum* (1) *M. avium* (type 2) (1) *M. paratuberculosis* (1) Not identified (1)
Drying bed sludge	23	8 *M. fortuitum* (6) Not identified (2)	0
Centrifuged sludge cake	9	3 *M. fortuitum* (1)	1 Not identified (1)
Final effluent	23	0	1 *M. paratuberculosis* (1)
Total	101	25	7

Strains were isolated only from samples with a pH range between 6·8 and 7·8. The majority (75%) were isolated from samples with a pH between 7·0 and 7·4.

Isolation of mycobacteria

Forty-nine strains of mycobacteria were isolated from 189 samples examined. Forty-one were identified as rapidly growing saprophytic types and eight as slow-growing, potentially pathogenic types. The samples from which these organisms were isolated and the identity of the isolates is shown in Tables 3 and 4. The majority of the strains, and particularly the slow-growing types (7/8), were isolated from plant D.

pH and total solids content of samples

The pH and total solids content of samples from plants C, D and F are shown in Table 5. The pH ranged from 5·3 to 8·2 and total solids from 0·01 to 88·0%.

DISCUSSION

Jones *et al.* (1980) reported on the isolation of salmonellas from 68% of samples of sewage, sewage sludge and final effluent from eight sewage treatment plants. With the possible exception of *Cysticercus bovis*, salmonellas present the most obvious and important hazard to animals allowed to graze pasture previously

Table 4. *Isolation of mycobacteria from treatment plant F*

Sample	No. examined	Number and identity of mycobacteria isolated: Fast-growers	Slow-growers
Settled sewage	22	5 *M. fortuitum* (2) *M. peregrinum* (2) *M. chelonei* (1)	0
Raw sludge	22	4 *M. fortuitum* (2) *M. peregrinum* (1) *M. gordonae* (1)	0
Anaerobically digested sludge	22	7 *M. fortuitum* (5) Not identified (2)	1 Not identified
Final effluent	22	0	0
Total	88	16	1

Table 5. *pH and total solids content of samples from treatment plants C, D and F*

Plant	Sample	pH: Range	pH: Mean	Solids content %: Range	Solids content %: Mean
C	Settled sewage	7·1–7·7	7·4	0·05– 0·4	0·14
	Raw sludge	5·3–6·1	5·8	3·0 – 8·0	5·6
	Centrifuged sludge cake	NT	—	14·5 –25·0	19·9
	Final effluent	7·1–7·7	7·4	0·05– 0·5	0·14
D	Settled sewage	6·9–8·2	7·5	0·03– 0·2	0·1
	Raw sludge	5·7–6·9	6·4	2·0 – 7·5	4·4
	Drying bed sludge	NT	—	16·0 –88·0	28·6
	Centrifuged sludge cake	NT	—	16·0 –26·0	20·2
	Final effluent	7·0–7·8	7·4	0·02– 0·2	0·07
F	Settled sewage	7·0–7·6	7·3	0·01– 0·3	0·1
	Raw sludge	5·5–6·1	5·8	1·5 – 7·0	3·8
	Anaerobically digested sludge	7·0–7·4	7·2	0·5 – 3·5	2·3
	Final effluent	6·2–7·5	7·1	0·01– 0·35	0·1

NT = not tested.

dressed with sewage sludge. Jones *et al.* (1980) concluded that sewage sludge should not present a greater hazard than farm slurries if sensible grazing restrictions are observed. However, the possibility of diseases being transmitted by the application of sewage sludge to pasture land could not be discounted entirely, particularly for several other bacterial pathogens which may gain access to sewage. The processes used in the treatment of sewage are extremely efficient in reducing the number of bacteria of enteric origin. Less than 1 % of coliforms present in raw sewage remain viable in final effluent or processed sludges (Table 1) and it is the number of pathogenic organisms which determine the dangers associated with these wastes.

Infections with *Brucella abortus* could be transmitted should this organism gain access to sewage. The sewage plant examined for brucellas in this survey was chosen since it received sewage from a large cattle market held weekly. *B. abortus* has been shown to survive in cattle slurry for a similar period to salmonellas (Rankin & Taylor, 1969) but was not isolated from slurry (Jones & Matthews,

1975) or waste from dairy factories (Jones, Bew & Gammack, 1975) during surveys in which salmonellas were successfully isolated. This may be a reflection of the difficulty of isolating *B. abortus* from a contaminated environment rather than an indication of the absence of the organism. However, the greatest risk of infection with this organism is from products of abortion (Wray, 1975) and the possibility of spread of brucellosis in sewage sludge will, of course, cease as the eradication scheme for this organism takes effect.

Similarly, the most important method of spread of enteropathogenic strains of *E. coli* is direct animal-to-animal contact and the isolation of 11 possibly enteropathogenic strains in this survey does not necessarily indicate that sewage sludge may be responsible for the spread of these organisms. It may, however, contribute to the contamination of the environment, particularly by transmitting strains from one farm to another.

Small mammals, particularly rodents, may be the principal reservoir of leptospirosis (Twigg, Cuerden & Hughes, 1968). Leptospires may survive for considerable periods in moist environments and may even multiply in aerated cattle slurry (Will & Diesch, 1972). They were not isolated from cattle slurry in Britain (Jones & Matthews, 1975) and Diesch, Pomeroy & Allred (1971) found difficulty in re-isolating *Leptospira pomona* seeded into a model oxidation ditch. Thirty strains were isolated during the survey reported here but only one, identified as *Leptospira tarassovi*, is considered as a possible pathogen. The identification of this strain will be the subject of a future publication. It is interesting that this strain was isolated from a large urban sewage plant (plant F) and not from the plant (plant D) which receives sewage from a cattle market.

Leptospires were isolated only from samples within a narrow pH range. The composition of sewage will vary considerably depending upon its origin and methods used for treatment (Table 5) and it is to be expected that various organisms may only be isolated from wastes of a certain composition. The composition of the sample may also determine the ease with which an organism will be isolated. It would, for example, be easier to isolate a pathogen from final effluent with total solids of 0·01 % than from a sludge with total solids of 88·0 % (Table 5) if the pathogen occurred in the same concentration in both, since there would be a larger number of contaminants in the sludge with its high solids content.

In contrast to the leptospire isolate, seven of the eight potentially pathogenic mycobacteria were isolated from plant D, perhaps reflecting the high proportion of animal waste received by this plant. Three of the pathogenic mycobacteria were identified as *Mycobacterium paratuberculosis*, the causative organism of Johne's disease in cattle, and one as *M. avium* type 2 which has also been shown to cause disease in cattle (Matthews, personal communication). *M. paratuberculosis* has been shown to survive for up to 246 days in infected faeces (Lovell, Levi & Francis 1944) and Rankin & Taylor (1969) considered that young animals grazing pasture contaminated with *M. paratuberculosis* within a year of slurry spreading are at risk. On the evidence of the isolations reported here the same restriction should perhaps be placed on allowing young stock to graze pasture spread with sewage

sludge. This may be particularly true in areas of the country where *M. bovis* is still endemic in the cattle population since this organism may also be expected to gain access to sewage.

However, it is possible that the number of mycobacteria contained in sludge following treatment is low, the majority in this survey being isolated from raw sludge, and if sensible grazing restrictions are observed the danger of infection is probably slight.

Bacillus anthracis was not isolated from the sewage plant (plant C) which received effluent from two tanneries but two organisms were recovered which closely resembled *Bacillus anthracis*, thus indicating that, had the organism been present in large numbers, it would have been isolated. However, the isolation methods available are insensitive and the possibility of small numbers of *Bacillus anthracis* being present cannot be excluded. This organism is known to survive in soil for as long as 60 years (Wilson & Russell, 1964) and thus it is possible that the continued application of sewage sludge from treatment plants receiving tannery effluent may lead to a build-up of this organism in the soil. It may accordingly be wise to limit the quantities of such material applied to pasture land. This is, however, a restraint which may also apply to the spreading of materials such as bonemeal on agricultural land, and sewage sludge may present a minimal hazard compared to other possible sources of environmental contamination.

Potentially pathogenic bacteria belonging to a number of genera have been isolated during the survey reported here and elsewhere (Jones *et al.* 1980). The concentration of such organisms is greatly reduced by sewage treatment processes and the spreading of sewage sludges on agricultural land should present no greater hazard than the spreading of animal slurries if sensible grazing restrictions are observed.

We are most grateful to Mrs Susan Knights and Miss Nicola Rolley (IRAD) for skilled technical assistance. We should also like to thank the Thames Water Authority for financing much of the work described, Mr V. H. Lewin, Mr D. L. Redhead, Mr B. J. E. Hurley, Mr P. E. Norris and Dr D. W. Johnstone of TWA for helpful advice and criticism, and the staff of the Authority who collected the samples of sewage examined.

We are also grateful to the Director of Scientific Services (TWA) for permission to publish.

REFERENCES

BUCHANAN, R. E. & GIBBONS, N. E. (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.

COUSINEAU, J. G. & MCKIEL, J. A. (1961). *In vitro* sensitivity of *Leptospira* to various antimicrobial agents. *Canadian Journal of Microbiology* **7**, 751.

DIESH, S. L., POMEROY, B. S. & ALLRED, E. R. (1971). Survival and detection of leptospires in aerated beef cattle manure. *Proceedings of the International Symposium on Livestock Waste, St Joseph. Michigan.* American Society of Agricultural Engineering.

ELLINGHAUSEN, H. C. JR. & MCCULLOUGH, W. G. (1965). Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. *American Journal of Veterinary Research* **110**, 45.

JOHNSON, R. C. & ROGERS, P. (1964*a*). Differentiation of pathogenic and saprophytic leptospires. *Journal of Bacteriology* **87**, 422.

JOHNSON, R. C. & ROGERS, P. (1964*b*). Differentiation of pathogenic and saprophytic leptospires with 8-azoguanine. *Journal of Bacteriology* **88**, 1618.

JOINT F.A.O./W.H.O. EXPERT COMMITTEE ON BRUCELLOSIS (1958). Third Report, Annex 10. Technical Report Series No. 148. 50. World Health Organization.

JONES, P. W., BEW, J. & GAMMACK, D. B. (1975). An investigation into the potential hazard to animal health of effluent sludge from dairy factories. *Journal of Hygiene* **75**, 143.

JONES, P. W. & MATTHEWS, P. R. J. (1975). Examination of slurry from cattle for pathogenic bacteria. *Journal of Hygiene* **74**, 57.

JONES, P. W., RENNISON, L. M., LEWIN, V. H. & REDHEAD, D. L. (1980). The occurrence and significance to animal health of salmonellas in sewage and sewage sludges. *Journal of Hygiene* **84**, 47.

LOVELL, R. G., LEVI, M. & FRANCIS, J. (1944). Studies on the survival of Johne's bacilli. *Journal of Comparative Pathology* **54**, 120.

MATTHEWS, P. R. J., COLLINS, P. & JONES, P. W. (1976). Isolation of mycobacteria from dairy creamery effluent sludge. *Journal of Hygiene* **76**, 407.

MORRIS, E. J. (1955). A selective medium for *Bacillus anthracis*. *Journal of General Microbiology* **13**, 456.

PEARCE, T. W. & POWELL, E. O. (1951). A selective medium for *Bacillus anthracis*. *Journal of General Microbiology* **5**, 387.

RANKIN, J. D. & TAYLOR, R. J. (1969). A study of some disease hazards which could be associated with the system of applying cattle slurry to pasture. *Veterinary Record* **85**, 578.

SOJKA, W. J. (1965). Escherichia coli in *Domestic Animals and Poultry*. Review series no. 7. Commonwealth Agricultural Bureaux, Farnham Royal: Commonwealth Bureau of Animal Health.

STUART, P. (1965). Vaccination against Johne's disease in cattle exposed to experimental infection. *British Veterinary Journal* **121**, 289.

TWIGG, G. I., CUERDEN, C. M. & HUGHES, D. M. (1968). Leptospirosis in British wild mammals. *Symposium of the Zoological Society, London* **24**, 75.

WILL, L. A. & DIESCH, S. L. (1972). Leptospires in animal waste treatment – animal health problem? *Proceedings of the 76th Annual Meeting, Animal Health Association.*

WILSON, J. B. & RUSSELL, K. E. (1964). Isolation of *Bacillus anthracis* from soil stored 60 years. *Journal of Bacteriology* **87**, 237.

WRAY, C. (1975). Survival and spread of pathogenic bacteria of veterinary importance in the environment. *Veterinary Bulletin* **45**, 543.