

THE ISOLATION AND IDENTIFICATION OF HAEMOPHILUS SPP. FROM UNUSUAL LESIONS IN CHILDREN

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From a high proportion of children sent to hospital *H. influenzae* can be isolated if suitable culture media are used. A number of *H. influenzae* strains were isolated from unusual sites, such as (1) blood cultures after tonsillectomy or tonsillotomy in five cases; (2) urine or the urinary tract in eight cases; (3) the lumen of appendices removed at operation in 11 cases (4%); (4) osteomyelitis or pyarthrosis in six cases; (5) miscellaneous infections including two perianal abscesses, three cases of paronychia, one infected thyroglossal cyst, and several skin infections.

It is suggested that infections of the skeletal system and the urinary tract arise from haematogenous spread of *H. influenzae*, as demonstrated by positive blood cultures after tonsillectomy and in two cases of skeletal infection. Infection of the appendix, perianal abscesses, paronychia, and skin infections probably arise by the direct route, either by immediate contact or by passage of viable organisms through the alimentary canal.

The routine bacteriological techniques that are used in the Birmingham Children's Hospital allow *Haemophilus influenzae* to be identified in any specimen except faeces. The species of *Haemophilus* that are the subject of this communication were isolated from children. There was no opportunity to examine similar material from adults. The purpose of this paper is to draw attention to the presence of species of *Haemophilus* in sites and lesions where these organisms are not usually expected or found.

Methods and Materials

Method of Isolation.—Specimens of pus, from any site, were seeded on heated blood (chocolate) agar plates, as well as on the "composite" blood agar plate described by Rogers and Heslop (1948). Heated blood agar plates were prepared by adding 10% of oxalated horse blood to heart infusion agar at about 60° C.; after gentle mixing, the temperature was raised slowly to 75–80° C. and maintained at that temperature for 10 minutes. (The colour of the mixture should then be that of milk chocolate and there should be no gross granularity or clumping.) The molten mixture was again mixed gently and poured into Petri dishes.

All inoculated heated blood agar plates were placed in large tin containers inside which a lighted small

wax candle or night light was placed to supply a higher CO₂ tension than found in normal atmosphere. The lid was sealed to the tins by a $\frac{3}{4}$ in. wide rubber elastic band. Pneumococci may require increased tension CO₂ for growth (Gladstone and Fildes, 1940; Fleming, 1941), and the presence of this gas does not impede the isolation of *H. influenzae*. This simple device was employed for every routine culture normally incubated aerobically, except those on desoxycholate citrate agar, MacConkey's, Hoyle's tellurite, or Sabouraud's media.

Early subcultures of strains of *H. influenzae* from an unusual situation were sent to Leeds for detailed identification, and typing where appropriate.

For the determination of X and V requirements, an autoclaved fresh or heated blood agar and an autoclaved yeastrel agar plate were used.

In the autoclaved blood agar plate the heat-labile V factor has been destroyed, while the heat-stable X factor is still present. Autoclaved yeastrel agar, as prepared for colony counts of water supplies (Ministry of Health, 1956), contains neither X nor V. In our experience it is the only medium consistently free from any trace of X.

Both plates were seeded evenly with the *Haemophilus* strain under test. A staphylococcus was then streaked halfway across the inoculated area. Strains dependent on X factor alone grow all over the inoculated area of the autoclaved chocolate agar plate, while on the same medium strains requiring

either V alone, or both X and V, do not grow except as satellite growth in the vicinity of the staphylococcus streak. On the yeastrel agar plate only V-dependent strains show satellitism near the staphylococcus streak. Strains requiring either X only, or both X and V, show no growth at all on yeastrel agar.

Type Identification.—For type identification, simple slide agglutination with the six type sera a to f was employed as a screening test. Floccular precipito-agglutination, similar to that seen with pneumococcus types and their sera, occurs almost instantaneously. However, a slower, granular, non-type-specific agglutination may occur with some sera and strains and is probably due to somatic antigens and their corresponding antibodies. These non-specific reactions make the slide agglutination technique less than 100% reliable, and it is therefore essential to obtain confirmation by the Neufeld technique, or, if preferred, by the formal tube agglutination method.

For the Neufeld technique, Levinthal broth cultures should not be older than five or six hours, at which time capsules are fully developed, and type-specific polysaccharides from them have not yet gone into solution in the culture medium. The age of the Levinthal broth culture can be reduced considerably if 1–2 ml. of the medium is pre-warmed to 37° C. before inoculation from an overnight chocolate or Levinthal agar plate immediately after the latter has been removed from the incubator. In this way the lag period is obviated. Capsules are well developed when the Levinthal broth culture shows the first signs of turbidity, i.e., after one or two hours if pre-warmed broth is used. Then a hanging drop preparation is made with (a) a loopful of the culture, (b) a loopful of type-specific *H. influenzae* antiserum, and (c) a discharged loop of Loeffler's methylene blue. The unstained capsules around the stained bacillary bodies are well defined when viewed through a microscope with corrected tube length. Cross reactions between *H. influenzae* types do not occur with the Neufeld technique or the formal tube agglutination.

Specimens.—Immediately before a tonsillectomy or tonsillotomy, a tonsillar swab and a blood culture were taken and another blood culture 3–4 min. after the operation. The blood samples were run into liquid and were then transferred to a 0.15% glucose-

1% peptone-infusion broth (Stuart, 1948). The removed tonsillar tissue was placed in a sterile Petri dish and the surface of the tissue was sterilized by searing. Then an incision was made with a sterile scalpel through the surface area thus sterilized and the fresh inner surface and depths of the crypts, exposed under sterile conditions, were cultured.

A few of the urinary strains were isolated from "clean" mid-stream specimens, but if *H. influenzae* was found in such circumstances a catheter specimen was taken, or at least a sample of urine obtained after scrupulous cleaning of the skin surrounding the meatus; from female children, a vaginal swab was taken at the same time.

Results

The strains of *H. influenzae* isolated in this way are grouped under headings according to the sites in which they were found.

Blood Cultures Immediately Before and After Removal of Tonsils.—Thirty-eight patients were examined, of whom 19 had their tonsils removed by tonsillectomy. None of the pre-operative blood cultures grew any pathogens. Five of the post-operation blood cultures grew *H. influenzae* in pure culture.

Although cultures from the tonsils usually yielded a mixed growth of pneumococci, haemolytic streptococci, and other mouth organisms besides *H. influenzae*, only *H. influenzae* was grown from the corresponding blood cultures. Four of the five strains were sent to Leeds for examination; three were not capsulated. One was a type b strain; both the pre-operative throat swab and the cultures from the tonsils of this child provided mixed growths which included non-capsulated *H. influenzae*. All colonies isolated from the tonsils and the throat of this case looked alike, but the presence of a few mucoid colonies in the cultures cannot be excluded, as a subculture from a single colony thought to be representative had been sent to Leeds for further examination.

TABLE I
ISOLATION OF ORGANISMS FROM BLOOD CULTURES AFTER TONSILLOTOMY AND TONSILLECTOMY

Operation	No. of Cases	Blood Culture					
		Pre-operative			Post-operative		
		Growth of			Growth of		
		Sterile	Coagulase-negative Staphylococci	<i>H. influenzae</i>	Sterile	Coagulase-negative Staphylococci	<i>H. influenzae</i>
Tonsillectomy	19	18	1	0	15	1	3
Tonsillotomy	19	17	2	0	16	1	2
Total	38	35	3	0	31	2	5

TABLE II

ISOLATION OF *H. INFLUENZAE* FROM TONSILS OF CASES IN WHICH THE ORGANISM WAS PRESENT IN THE BLOOD STREAM POST-OPERATIVELY

No. of Colonies of <i>H. influenzae</i> Grown	Pre-operative Tonsillar Swab	Post-operative Cultures from Tonsils
None	1	2
A few	2	2
Many	2	1

The results of the tonsillar cultures are summarized in Tables I and II. There were three positive blood cultures after tonsillotomy and two after tonsillectomy.

Urinary Cultures.—Eight children have been found to have urines infected with *H. influenzae*: none has been infected with *H. parainfluenzae*. Two of the eight children, from whom urine strains of *H. influenzae* were isolated, had a recent and violent injury. Five others had congenital abnormalities of the urinary tract. In two a calculus of the pelvis of the kidney was found and one of these two children also had hydronephrosis. *H. influenzae* was grown twice from catheter specimens and three times from clean specimens of one of these children (No. 3 in Table III).

Clinically it was noteworthy that sometimes *H. influenzae* was isolated from the urine of a child attending the hospital for the first time with what was thought to be an attack of uncomplicated pyelitis. Subsequently every one of these children returned with repeated urinary tract infections. On further investigation each of the children was found to have either renal calculi or congenital

abnormalities of the urinary tract. The results are summarized in Table III.

One case deserves particular mention.

This boy had had both ureters transplanted into the colon at the age of 11 months because of a congenital ectopia vesicae. Four years later a stenosis of the right urethrocolic anastomosis developed and this was relieved by a plastic operation. Then a hydronephrosis of the right kidney developed and persisted. Six years later, at the age of 10, the boy complained of pain in the right renal region. Radiographs showed that a calculus was present in the renal pelvis. The calculus was removed to preserve the kidney and to allow the ureter to be re-implanted in the colon at a later date. When the calculus was exposed, a swab was taken from the turbid fluid in the renal pelvis. This yielded a heavy growth of type 5 pneumococci and a moderate number of *H. influenzae*. Cultures of the faeces failed to grow either of these organisms, although *E. coli*, green-forming non-haemolytic and Lancefield Group D haemolytic streptococci were isolated.

Cultures from Lumen of Appendices Removed at Operation.—For several years past, routine cultures have been made from the contents of appendices removed at operation for the purpose of identifying any *Salmonella* or *Shigella* strains (Rogers, 1957; White *et al.*, to be published). At the same time, the contents of the appendices were treated as specimens of pus even if pus was absent, in an attempt to culture pyogenic organisms.

Eleven strains (Table IV) of non-capsulated *H. influenzae* were found in 327 appendices investigated in this way (3.4%).

TABLE III
ISOLATIONS OF *H. INFLUENZAE* FROM URINARY TRACT

No.	Sex and Age	Abnormalities	Catheter Specimen	Growth of <i>H. influenzae</i>	Remarks
1	F 3	Hydroureter Hydronephrosis	No	Numerous non-capsulated <i>H. influenzae</i> , few <i>E. coli</i>	Microscopically many <i>H. influenzae</i> in urine on first attendance at hospital. Further attacks of urinary infection were due to other organisms
2	M 3	Imperforate anus; rectovesicular fistula; permanent colostomy	Yes, through indwelling catheter	Heavy, pure	Had had many attacks of tonsillitis, very ill on admission
3	F 4	Bilateral hydroureter, hydronephrosis	Yes	Heavy, pure, non-capsulated	Grown repeatedly from catheter specimens. Patient's serum without agglutinins against own <i>H. influenzae</i> strain
4	F 5	Chronic pyelonephritis; staghorn calculus	..	Heavy, together with a few coliform bacilli and occasional <i>Staph. albus</i>	<i>H. influenzae</i> predominant
5	F 12	Bilateral hydronephrosis	..	Heavy, pure, non-capsulated	Of many attacks of pyelitis only one due to <i>H. influenzae</i>
6	M 11	Bilateral hydronephrosis; transplanted ureters; calculus in pelvis of kidney	Direct	Heavy, non-capsulated, mixed with equal number of pneumococcus type 5 colonies	Nephrostomy specimen; for details see text
7	F 9	None; see "Remarks"	No	Moderate, together with a few coagulase-positive <i>Staph. aureus</i> colonies	Heavy 6 ft. fall 3 days before admission, heavy haematuria for 10 days; recovered when treated as pyelitis
8	F 6	Injury in kidney region	..	Heavy, together with a few colonies of <i>E. coli</i> and <i>Staph. albus</i>	Microscopically many pus cells and <i>H. influenzae</i> in urine; developed dysuria

TABLE IV
BACTERIAL FLORA OF APPENDICES REMOVED FROM CHILDREN

Year	<i>E. coli</i> *	Non-lactose Fermenting Bacilli not Salmonellae or Shigellae	Salmonellae	Shigellae	Streptococci other than β -Haemolytic	Lancefield Group F Haemolytic Streptococci	Pneumococci	<i>H. influenzae</i>	Staphylococci	No Gram-negative Bacilli Isolated	No. Appendices Cultured
1956 ..	146	44	1	12	103	21	3	2	15	13	170
1957 ..	79	14	0	2	31	14	4	2	3	4	90
1958 ..	64	10	1	0	29	11	5	7	6	3	67
1959 ..	45	44	0	2	30	5	2	4	0	2	49
Total	334	72	2	16	193	51	14	15†	24	22	376
% of total	88.8	19.1	0.5	4.3	51.3	13.6	3.7	4.0	6.4	5.9	

* One strain each of specific serological types O.125, O.119, O.111. † One strain type f.

Table IV also shows not inconsiderable yearly fluctuations of the species isolated from the lumina of appendices.

Pneumococci were present in 12 specimens (3.7%) often as the only other organism besides *H. influenzae*. The satellite phenomenon and absence of antagonism between the two organisms was noted consistently. The types of pneumococci found were 4 (1), 6 (2), 9 (3), 19 (2), 23 (2), 34 (1), and 35 (1), the figures in brackets indicating the number of strains of each type isolated.

Pus in Sites Unconnected with Respiratory, Alimentary, or Urinary Tracts.—Abscesses and osteomyelitis and pyarthrosis furnished the specimens.

Abscesses.—Non-capsulated strains of *H. influenzae* were isolated in nearly pure culture from two perianal abscesses, where they probably arrived via the gastrointestinal tract in the same way as the organism was thought to have reached the lumina of the appendices. Pure growth of *H. influenzae* was obtained from pus of an infected thyroglossal cyst.

A comparatively rare species of the genus *Haemophilus*, *H. parahaemolyticus*, which is sometimes found in acute tonsillitis and subacute endocarditis (Pittman, 1953), was isolated, together with coagulase-positive *Staphylococcus aureus* and *albus*, from an abscess under the left mandible in a boy aged 15 months. The dental surgeon who incised the abscess stated: "The infection obviously comes from the mandible as bare bone was easily palpable through the incision."

In 1929, Fleming reported the isolation of *H. influenzae* from septic wounds. Similarly we succeeded on several occasions in its isolation in mixed culture from infected skin, also from the pus of paronychia. One was from the finger of a boy aged 6 years, when non-capsulated *H.*

influenzae was present together with *Staphylococcus aureus* and *Strep. pyogenes*. In another similar case, a boy of 4 years, non-capsulated *H. influenzae* was isolated together with haemolytic streptococci of Lancefield's group F, which normally is a commensal in the nasopharynx (Long and Bliss, 1934; Hare and Maxted, 1935). It is interesting to note that both these boys were finger suckers. A third paronychia yielded a mixed culture of *Staphylococcus aureus* and non-capsulated strain of *H. influenzae*.

Osteomyelitis and Pyarthrosis.—There were three cases of osteomyelitis of long bones, one with osteomyelitis of the tibial end of the knee and two with pyarthrosis infection in the hip joint, and also one child with osteomyelitis of the os calcis. Two children had an acute pyarthrosis of the hip, in all six cases with involvement of bones or joints. One of the cases of pyarthrosis of the hip was caused by a non-capsulated strain of *H. influenzae*: and a second non-capsulated strain was isolated from a case of osteomyelitis of long bone, but in this case the presence of a capsule initially cannot be ruled out because, owing to the long holiday period, the strain was subcultured for several weeks before it could be typed. From all other cases, *H. influenzae* type b was isolated, and this type was also recovered from the blood cultures of two of the children, one with pyarthrosis of the hip, the other with osteomyelitis of the tibial end of the knee joint.

Discussion

From these observations the question arises how *H. influenzae*, which is a pathogenic organism of the upper, and sometimes the lower, respiratory tract, could establish itself in the situations from which it was isolated. The literature on *H. influenzae* meningitis and epiglottitis makes it clear

that invasion of the blood stream by *H. influenzae* is not such a rare occurrence as one might be led to believe (Zinnemann, 1960). Once *H. influenzae* has been carried by the blood stream to a tissue with lowered local resistance, establishment of a focus of infection is a possible consequence.

Thus the strains of *H. influenzae* isolated from urinary tract infections in children with either congenital abnormalities of the urogenital system or with stone formation in the pelvis of the kidney cannot have reached these loci minoris resistentiae except as a result of a transient bacteraemia. The work of Rocha, Guze, Freedman, and Beeson (1958) and Freedman and Beeson (1958) offers an explanation of unusual susceptibility of the kidney to infection and in particular of the increased vulnerability of damaged kidneys. They showed that when the renal medulla was damaged the nephrons draining through that part of the kidney were particularly susceptible to a pyogenic infection if a bacteraemia recurred. Albright, Dienes, and Sulkowitch (1938) also reported repeated isolation of *H. influenzae* from the urine of two adults suffering from pyelonephritis and nephrocalcinosis. Thus the identification of *H. influenzae* in an infected urine indicates the need for detailed investigation of the patient to exclude the presence of abnormalities. The case in which turbid fluid obtained at nephrostomy contained non-capsulated *H. influenzae* and a pneumococcus type 5 emphasizes the pathogenesis of such infections of the urinary tracts, because there can be no doubt that this specimen came from the kidney.

One of the ways in which such infections can start has been demonstrated by the finding of *H. influenzae* in blood cultures after removal of the tonsils. The isolation of *H. influenzae* from blood cultures taken after such operations was somewhat unexpected, because Okell and Elliott (1935) never isolated *H. influenzae* in any of their positive blood cultures before or after tooth extractions, although they isolated more than one organism on several occasions, and Ross and Rogers (1943) isolated streptococci but not *H. influenzae* after root canal instrumentation of infected but viable teeth. Our findings were all the more surprising because a selective bacteraemia must have taken place, for on several occasions only a few colonies of *H. influenzae* were present in either pre- or post-operative tonsillar swabs together with other more numerous micro-organisms. However, *H. influenzae* could always be isolated from either the pre- or post-operative tonsillar swab or culture when it was found in the blood stream after tonsillectomy. We know only

one report in the literature that bears on this point, a case reported by Clarke (1912), of a girl aged 13 with chronic, intermittent otitis media due to *H. influenzae*, who developed septicaemia and osteomyelitis following tonsillectomy.

The case of Clarke also demonstrated for the first time that infection of the skeletal system with *H. influenzae* can be traced back to bacteraemia. There are only a few references in the literature to osteomyelitis and pyarthrosis due to *H. influenzae*. The 31 cases reported between 1899 and 1938 have been reviewed by Weaver and Sherwood (1938), who added two cases of their own. Both these, one an adult coloured woman and one a coloured child of 18 months, had pyarthrosis. In 1947, Hirsch and Nassau described one case with osteomyelitis and abscesses of the subcutaneous tissue; this was due to untyped *H. influenzae* and followed an acute febrile pharyngitis within four days. In the era before the advent of antibacterial drugs Weaver and Sherwood (1938) could point out that when osteomyelitis or pyarthrosis was preceded, accompanied, or followed by meningitis due to *H. influenzae* survival could not be expected, whilst practically all cases suffering only from *H. influenzae* infection of the skeletal system recovered, usually with a high degree of mobility. Antibiotics and sulphonamides have changed the prognosis of meningitis due to *H. influenzae* so much that nowadays a case fatality rate of more than 4% is regarded as a reason for a review of therapeutic procedures (Zinnemann, 1960).

It is suggested that a different mechanism operates when *H. influenzae* establishes itself as the cause of infection in an appendix or causes a perianal abscess. Although infection of the blood stream cannot be ruled out, the direct route via the upper respiratory and the alimentary tracts seems more plausible, particularly if one assumes that micro-organisms enveloped in a ball of highly viscous mucus or mucopus, which is a characteristic of *H. influenzae* infections, may pass the zones of gastric and duodenal digestion unharmed. Fleming (1929) previously isolated *H. influenzae* from the faeces. Two groups of Australian workers have published evidence to support their contention that oropharyngeal secretions reached the intestines. Anderson and Langford (1958) isolated three strains of *H. parainfluenzae* out of 12 jejunal specimens aspirated from normal children and three from 15 children with coeliac disease. Bishop and Allcock (1960) examined fluid aspirated from the small intestine above obstructions; from 27 patients they isolated two strains of *H. parainfluenzae* and *H. aphrophilus*. Neither of these groups isolated any strain of

H. influenzae or *H. parahaemolyticus*. Hare and Maxted (1935) recovered *Streptococcus pyogenes* from the faeces of cases of scarlet fever, which findings tend to support the assumption of respiratory organisms passing through the alimentary tract in a viable state.

The isolation of *H. influenzae* from a skin lesion was reported by Fleming in 1929; he used penicillin incorporated in culture media for its isolation. Penicillin was not a constituent of the routine nutrient media used in this investigation, and it is possible that some strains from this site have been missed. On the special blood agar plates used (Rogers and Heslop, 1948), the solution of 1 in 10,000 gentian violet, which is spread over the outer segments, nearly always inhibits *H. influenzae*. This inhibition is helpful in the identification of *H. influenzae*. Inhibition of *H. influenzae* also takes place at a 50 times higher dilution of gentian violet, which is the concentration of the dye in Garrod's blood agar plate (1942). These observations are not in accord with the findings of Bernstein and Loewe (1919) or of Masters, Brumfitt, Mendez, and Likar (1958), who used gentian violet in solid media to facilitate the isolation of *H. influenzae*.

It is important that the isolation of *H. influenzae* in unusual sites should be seen in perspective. For

this reason a survey of the number of strains isolated from other routine specimens during 1957 at the Children's Hospital is given in Table V.

Only the 10 strains of *H. influenzae* isolated from cerebrospinal fluid were typed; all were of type b. The other 582 strains were not so examined. These figures are presented to show how frequently *H. influenzae* is found in children attending hospital.

If routine cultures are made with a technique that permits the growth of *H. influenzae*, then the results obtained in Birmingham and reported here probably would be a common experience. In this sense we do not regard our findings as exceptional.

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TABLE V

SURVEY OF NUMBER AND PERCENTAGE OF STRAINS OF *H. INFLUENZAE* ISOLATED FROM ROUTINE SPECIMENS DURING 1957 AT THE CHILDREN'S HOSPITAL, BIRMINGHAM

Type of Examination	Total Specimens	<i>H. influenzae</i> Present		<i>H. influenzae</i> Predominant Organisms		
		No. Isolated	%	No. Isolated	% of Total Isolations	% of All Specimens
Throat	863	389	45	76	20	9
Nose	136	71	52	36	50	22
Ear	238	63	26	25	40	1
Conjunctiva ..	61	24	39	11	46	18
Antra	72	35	48	16	46	22
Cerebrospinal fluid	24	10	42	10	100	42