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Studies on the microbial flora in the air of submarines and the nasopharyngeal flora of the crew

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SUMMARY

Airborne bacteria surveys in nuclear submarines show that the total microbial load was maintained at satisfactorily low levels during prolonged patrols despite factors which were expected to increase this form of pollution.

The isolation rates of *Staphylococcus aureus* and *Neisseria meningitidis* from nasal and nasopharyngeal swabs respectively, together with the serum antibody titres to *Mycoplasma pneumoniae*, before and after patrols suggested that the transmission of these organisms between individuals was not much increased by patrol conditions. The finding of higher numbers of airborne gram-negative rods and bodily contamination by enterobacteria is frequently reported in submersibles but does not appear to cause major outbreaks of illness in nuclear submarine crews.

INTRODUCTION

The introduction of nuclear submarines and space capsules in which men live in sealed environments brought many new problems in atmospheric control (Ebersole, 1960; Lambert, 1970), especially in situations where even minor illness can determine the success or failure of a mission.

The microbiological implications of confining men for several weeks in submarines with continuously recycled air systems were examined by Watkins *et al.* (1970) who emphasized the need for systemic surveillance and pre-patrol screening procedures. They showed that in addition to those factors normally associated with military populations in barracks and ships, many others affect the bacteria load in a submarine. Some components of the air revitalization system are bactericidal, though not specifically designed for this purpose. The catalytic burners used to remove carbon monoxide, for example, operate at around 500° F. and it is worthy of note that Bourdillon (1945) suggested heating air to this temperature as a method of destroying airborne micro-organisms in ships when respiratory tract infections caused concern during World War II.

Watkins and his colleagues found that a major factor influencing the concentration of airborne bacteria was the inboard venting of excess air from the sewage tanks following their discharge by high pressure air at depth. After venting, transitory counts of the order of 10^5 bacteria per cubic foot of air were found, and

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organisms of faecal origin were recovered in a high proportion of throat and nasal swabs from the crews.

In view of these findings, a small working party was set up at the start of the British Polaris submarine programme to assess the risks of infection in submarine personnel, and if necessary to advise on measures to reduce them. Four main objectives were established, namely:

1. To assess the airborne microbial contamination of submarines during prolonged patrols.

2. To follow the spread of indicators of infection among the crews, for example, *Staphylococcus aureus* in nasal swabs, *Neisseria meningitidis* in nasopharyngeal swabs and serum antibody titres to infective agents such as *Mycoplasma pneumoniae*.

3. To provide medical officers accompanying the patrols with materials and directions for the collection, identification and preservation of organisms isolated during patrol for subsequent study in shore reference laboratories.

4. To analyse the reasons for sick bay attendance on patrols.

MATERIALS AND METHODS

Environmental studies

The Bourdillon (Bourdillon, Lidwell & Thomas, 1941) slit sampler was used to determine the number of airborne particles carrying viable bacteria: total counts were estimated from the resultant colonies on Oxoid blood agar base enriched with 5% horse blood or serum using a 2 min. (= 60 litres, $\simeq$ 2 cubic feet air) sample time. Counts of organisms of bowel origin were made on Oxoid MacConkey agar No. 2 using a 5 or 10 min. sampling time ($\simeq$ 5 or 10 cubic feet samples respectively).

Attempts were also made to estimate airborne concentrations of *Streptococcus salivarius* by the method of Willlams & Hirch (1950) and of *Staph. aureus* using various selective media (Harding & Williams, 1969) but these failed largely because of the difficulties of preparing or storing complex media on board the submarine.

After collection of the air samples, the resultant colonies were counted after 24 and 48 hr. incubation at 37° C. The results reported here were usually based on the 48 hr. count, but swarming colonies of *Proteus* or *Bacillus* species occasionally interfered and in these cases the 24 hr. counts were included. Attempts were made to identify the genera isolated with the limited range of simple tests available on board and random colonies were subcultured to maintenance media for subsequent identification in shore laboratories. The nomenclature and characterization tests of Cowan & Steel (1965) were used throughout.

Human studies

Nasal swabs for Staphylococcus aureus

Samples were collected on plain cotton-wool swabs, spread directly on salt mannitol agar plates and incubated at 37° C. for 18–24 hr. Presumptive *Staph. aureus* colonies were tested for coagulase production and positive strains phage

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typed and tested for sensitivity to penicillin, streptomycin, chloramphenicol, tetracycline and erythromycin using Oxoid Multodisks.

Nasopharyngeal swabs for Neisseria meningitidis

On early patrols, nasopharyngeal swabs were spread directly on heated blood agar plates which, after incubation for 18–24 hr. at 37° C. in 10 % carbon dioxide, were examined for characteristic colonies of *N. meningitidis*, the identity of which was then confirmed by standard methods. A 20 % isolation rate of *N. meningitidis* in the first complete Polaris crew examined was followed by very low rates in succeeding surveys and these were thought to be due to collection and technical difficulties.

The method which yielded the highest recovery rate and most consistent results was that in which samples were collected on cotton wool swabs supported on applicators bent at an angle of 30°. They were spread directly on prewarmed plates of Difco Mueller-Hinton agar containing 300 μg. vancomycin, 750 μg. colistin and 1250 units nystatin per 100 ml. Plates were incubated at 37° C. in 10 % CO_2 for 18–24 hr. and colonies of *N. meningitidis* further identified by standard methods prior to testing for sulphonamide resistance and antigenic typing (R. J. Fallon and P. H. Marsden, unpublished).

Nasopharyngeal swabs for total and differential colony counts of bacteria

During the studies on meningococcal carrier rates, large numbers of gram-negative rods, chiefly of the Enterobacteriaceae but also many pseudomonads, were isolated from nasopharyngeal swabs. Semi-quantitative studies were therefore carried out to determine the extent, frequency and duration of throat carriage of these organisms. The technique used was that in which soluble alginate swabs on flexible wires were taken by one observer, well mixed in Calgon–Ringer solution and plate counts made by the technique of Miles & Misra (1938). All colonies were differentiated into Gram-positive cocci and rods and Gram-negative cocci and rods, and random colonies of Gram-negative rods from a proportion of the samples were identified by standard biochemical methods.

Serum antibody titres to Mycoplasma pneumoniae

Surveys for antibodies to *Mycoplasma pneumoniae* were carried out on two crews by complement fixation tests using the lipid antigen of *M. pneumoniae* (Kenny & Grayston, 1965) in the method of Bradstreet & Taylor (1962) as modified by Grist, Ross, Bell & Stott (1966) and by metabolic inhibition tests (Taylor-Robinson, Purcell, Wong & Chanock, 1966).

RESULTS

Airborne bacteria

The results summarized in Tables 1 to 5 were derived from surveys in three Polaris submarines during eight patrols of up to sixty days in which the effects of duration of patrol, time of day and occupational area on the number of viable

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Table 1. *Effect of patrol time on the total airborne bacteria-carrying particle count. The mean colony count/ft.³ and standard error of the mean* (S.E.M.) *was derived from the indicated number of air samples after correction for the 2 ft.³ sample volumes*

	Number of air samples	Mean colony count/ft.³	S.E.M.
Before patrol	108	9·1	0·3
Weeks of patrol 1	102	11·0	0·4
2	48	15·5	0·9
3	88	16·0	0·8
4	76	16·2	0·9
5	46	15·8	0·8
6	—	—	—
7	56	15·9	0·9
8	30	15·8	1·2

Table 2. *Effect of patrol time on the number of airborne particles carrying organisms of bowel origin. The values were derived as for Table 1 except that correction was made for the 5 or 10 ft.³ sample volumes*

	Number of air samples	Mean colony count/ft.³	S.E.M.
Before patrol	40	0·52	0·03
Weeks of patrol 1	50	2·54	0·16
2	—	—	—
3	25	3·04	0·30
4	30	3·41	0·31
5	14	2·40	0·33
6	—	—	—
7	16	2·68	0·29
8	10	3·55	0·40

airborne bacteria are examined. There were no significant differences in the results between submarines or between the slit samplers used in these surveys.

There was a statistically significant increase in total airborne bacteria in the first week of patrol compared with the prepatrol period ($t = 3{\cdot}8$, $P < 0{\cdot}001$) and in the second patrol week compared with the first ($t = 5{\cdot}3$), $P < 0{\cdot}001$). There was no significant change during the remainder of patrol, the overall mean being 16 airborne bacteria-carrying particles per cubic foot ($\simeq 0{\cdot}45/m^3$) of air. The number of organisms of bowel origin was also significantly increased in the first week of patrol compared with prepatrol values ($t = 10{\cdot}1$, $P < 0{\cdot}001$) and remained higher throughout, but with more variations than in the total counts of airborne bacteria.

A steady increase in the number of airborne bacteria occurred during the working day and peak values were reached in the early evening when the majority of the crew were bathing, dining and taking part in social activities (Table 3). That higher counts were clearly associated with human activity is clear from Table 4 where the counts are very low in the relatively unoccupied working spaces.

The results of the post patrol identification studies are presented in Table 5 which summarizes the identity of some 3000 colonies subcultured to maintenance media during patrols. A little over 3% did not survive or were not identified.

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Table 3. *Effect of time of day on the total airborne bacteria-carrying particle count. The values were derived as in Table* 1

	Number of air samples	Mean colony count/ft.^{3}	S.E.M.
Midnight – 04.00	46	8·5	0·3
04.00 – 08.00	50	9·0	0·3
08.00–12.00	128	12·5	0·5
12.00–16.00	116	14·3	0·8
16.00–20.00	126	19·4	1·1
20.00 – Midnight	88	11·5	0·5

Table 4. *Total airborne bacteria-carrying particle counts in different areas of the submarine. The values were derived as in Table* 1

	Number of air samples	Mean colony count/ft.^{3}	S.E.M.
Accommodation Spaces			
Galley	45	14·8	0·8
Bunk space	52	13·5	0·5
Messes	125	14·2	1·0
Heads and bathrooms	150	16·9	1·0
Average for overall accommodation spaces	372	15·3	—
Average for working spaces	90	5·2	0·15

Table 5. *Differential counts of airborne bacteria recovered from air samples during patrols*

Gram-positive species				Gram-negative species			
Spheres		Rods		Spheres		Rods	
Micrococcus	40·9 %	*Corynebacterium*	15·0 %	*Neisseria*	2·8 %	*Acinetobacter*	7·0 %
Staphylococcus	10·5 %	*Bacillus*	5·5 %			*Enterobacter*	6·5 %
Aerococcus	3·1 %					*Escherichia*	4·0 %
*Streptococcus**	2·9 %					*Proteus*	0·8 %
						Pseudomonas	0·6 %
						Alcaligenes	0·4 %
Total = 77·9 %				Total = 22·1 %			

* 62 % of these were *Strep. faecalis*.

Human studies

Staphylococcus aureus *in nasal swabs*

The nasal carriage rate of *Staph. aureus* and the proportion of strains resistant to the five antibiotics used routinely for testing are shown in Table 6. It is evident that although there were no significant changes in carriage rate during patrol, there was an apparent increase in the proportion of strains exhibiting antibiotic resistance.

There was no evidence of selection of particular phage types of *Staph. aureus* during patrols; there were 88 different phage types in 264 of the strains isolated before patrol with 27 strains untypable at 100 RTD and 56 different types in 150 strains isolated after patrol with 8 untypable.

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Table 6. *The carriage rate and number of resistant strains of* Staph. aureus *in nasal swabs from submarine crews*

	Before patrol	During patrol	End of patrol
Number of swabs examined	917	175	493
Number positive for *Staph. aureus*	291 (31·7)*	58 (33·1)	158 (32·0)
Number resistant to			
Penicillin G	91 (31·3)	30 (51·7)	85 (53·8)
Streptomycin	4 (1·4)	4 (~ 7)	7 (4·4)
Tetracycline	4 (1·4)	4 (~ 7)	9 (5·7)
Erythromycin	0	0	4 (2·5)
Chloramphenicol	2 (0·7)	1 (~ 2)	2 (1·3)

* Figures in parentheses indicate percentages.

Table 7. *Nasopharyngeal carriage rates of* N. meningitidis *and the proportion of sulphadiazine resistant strains*

	Before patrol	End of patrol
Number of swabs examined	266	127
Number of swabs positive for *N. meningitidis*	71 (26·7)*	36 (28·3)
Number of strains growing in the presence of sulphadiazine (mg./100 ml. medium)		
1·0 (resistant)	0	2 (~ 5)
0·1 (partially resistant)	7 (~ 10)	7 (~ 19)
0·01 (sensitive)	70 (~ 84)	25 (~ 69)
Not tested	4	2

* Figures in parentheses indicate percentages.

Neisseria meningitidis *in nasopharyngeal swabs*

The isolation rates of *N. meningitidis* from nasopharyngeal swabs and the proportion of strains resistant or partially resistant to sulphadiazine are presented in Table 7. This work is incomplete and the figures are insufficient to allow firm conclusions to be drawn; they are shown here because the relatively unchanged isolation rates and suggestive increases in sulphadiazine resistant strains were similar to those observed with *Staph. aureus*. Further surveys are being carried out and the results will be reported later (P. H. Marsden & R. J. Fallon, unpublished.)

Streptococcus pyogenes *in throat swabs*

Only 13 isolations of Lancefield group A β haemolytic streptococci were made from some 1300 throat swabs examined before and after patrols.

Semi-quantitative estimates of nasopharyngeal flora

Table 8 summarizes the results of the semi quantitative assay of the nasopharyngeal flora of two crews before and after a patrol. The frequency distribution curve is not normally distributed, being skewed to the right, and a statistical treatment of the results by log probit analysis indicates that there is no significant change in the number of nasopharyngeal organisms as a result of patrol. There is, however, a clear-cut difference in the type of organisms recovered at the end of

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Table 8. *Estimates of the number and type of nasopharyngeal organisms in submarine crews before and after a patrol. Values are based on the log of the probable number of organisms per swab from a Miles & Misra* (1938) *count, and the standard deviations* (S.D.) *by log probit analysis*

	Before patrol	End of patrol
Number of swabs examined	217	165
Viable organisms/swab $\times 10^3$ Log mean	22·5	26·5
Range (−2 S.D.)	16·0	18·8
(+2 S.D.)	40·5	47·8
Gram-positive cocci (mainly *Staph.*, *Strep.* and *Micrococ.* spp.)	58 %	20 %
Gram-positive rods (*Bacillus*, *Corynebacterium* spp.)	12 %	24 %
Gram-negative cocci (*Neisseria* spp.)	10 %	10 %
Gram-negative rods (Mainly *Haemophilus* spp. and enterobacteria)	10 %	46 %

patrol compared with the beginning, with a fall in the proportion of gram-positive cocci and a corresponding rise in gram-negative rods. The types of gram-negative organisms isolated were similar to those found in air samples and include *Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus* and *Pseudomonas* species. *Strep. faecalis* was also found frequently.

Serum antibody titres to Mycoplasma pneumoniae

M. pneumoniae antibodies were found in 84/412 (20·4 %) of pre-patrol sera and in 27/140 (19·3 %) post-patrol sera tested by complement fixation; in 195 pre-patrol sera tested by both complement fixation and metabolic inhibition (neutralization) tests, antibodies were found in 40 (20·5 %) by MIT, 36 (18·5 %) by CFT and 63 (32·3 %) by one or the other test. These rates were similar to those for civilian sera tested in the same laboratory (R. J. Fallon, unpublished data).

DISCUSSION

On the evidence of slit sampler counts of total airborne bacteria-carrying particles, microbial pollution of the submarine atmosphere remains at an acceptably low figure during long patrols and the air revitalization system can easily cope with the increases associated with human activity. The values compare favourably with those quoted by Williams, Lidwell & Hirsch (1956) in schools, offices and factories and are significantly lower than those reported by Ellis & Raymond (1945) in the overcrowded ships of World War II. The results presented here are not strictly comparable with those of Watkins *et al.* (1970) for U.S. Navy Polaris submarines since the latter used raised jet impingers to collect 20 min air samples in 5 % skim milk saline which were then frozen until the end of patrol for analysis in shore laboratories. Impinger counts are usually higher than those derived by slit sampler methods, but some of the U.S. Navy figures appear even higher than would be explained by the difference in methods and the occasional very high counts, of the order of 3×10^4 organisms per cubic foot of air ($\simeq 10^3$/m.^{3}),

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were not seen in the present study. The question arises whether multiplication of some organisms during storage and assay contributed to such high results.

The use of bacterial counts to indicate the hygienic state of the air in occupied spaces has been investigated for many years without a satisfactory conclusion being reached (Wilson & Miles, 1964; R. E. O. Williams, personal communication, 1968). Reid, Lidwell & Williams (1956) observed positive correlations between various respiratory infections in school children and general flora counts of their classrooms, but concluded that these relationships would not justify the use of total airborne bacteria counts as indices of the risk of infection. There is general agreement that in assessing the risk to the inhabitants of a closed space, it is not the total microbial load, but the number of airborne pathogens which is important. This number may be very small (Riley, 1957) and not detectable by normal sampling methods, it also varies for different species. Some workers have therefore used counts of 'indicator' organisms to assess the risks in the same way as the probable coliform count is used in water testing.

Table 2 shows that about one fifth of the airborne bacteria in a dived submarine are probably of bowel origin as indicated by MacConkey agar counts, and this is supported by the differentiation of organisms from the blood agar plates (Table 5).

The numbers and proportions of coliform organisms and enterococci are higher than in Williams's 1956 surveys but appear to be common in submerged vessels. Davies, Valentine & Feindler (1970), in detailed surveys of the water systems, human flora and general environment of the *Ben Franklin* submersible used as a space station analog, also observed widespread contamination with gram-negative rods, notably *Pseudomonas*, *Escherichia* and *Proteus* species.

There is general agreement that organisms of bowel origin can readily be recovered from the upper respiratory tract of the crews of these ships. In addition, the American workers found significant increases in enterobacterial contamination of the skin despite, in the case of the *Ben Franklin*, positive attempts to reduce it by antimicrobial soaps and treated garments. The shift to predominantly gram-negative organisms in the nasopharyngeal flora, reported in the *Ben Franklin*, is repeated in the British Polaris submarine crews. Whether these shifts are due to aerosols generated in the submarine or whether the ambient conditions (for example, raised carbon dioxide affecting tissue pH), favour survival of particular species is not yet clear, but it is likely that the former is the main factor. In repeated nasopharyngeal swabs from thirty individuals on patrol, for instance, the predominant organisms on one particular day were *Pseudomonas* species, but three days later *Strep. faecalis* was the most frequent isolate. Ten days later, *Proteus* and *Esch. coli* were recovered from the majority of the swabs whilst towards the end of patrol, *Pseudomonas* reappeared as the major component. It is of interest that on this patrol a minor outbreak of otitis externa was shown by pyocine typing to be due to the same strain of *Ps. aeruginosa* as that isolated from apparently healthy throats a few days earlier.

Survey of the spread of indicators of infection yielded largely negative, and therefore encouraging results. There was no increase in the nasal carriage rate of *Staph. aureus* and little or no evidence of selection of particular phage types of

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these organisms during patrols. It was occasionally possible to follow an individual strain and in the two main cases where this occurred it may be significant that medical staff appeared to be the originators. A penicillin resistant strain of *Staph. aureus,* phage type 29/52/52A/53/54/79/80/85 isolated from a medical technician at the start of one patrol was subsequently cultured at the end of patrol from four of his shipmates and from a further seven by the end of the next patrol. A similarly well defined strain was transferred from the doctor to some of his patients in another crew.

The isolation rates of *Staph. aureus* in nasal swabs and *Neisseria meningitidis* in nasopharngeal swabs and the serum antibody titres to *Mycoplasma pneumoniae* suggest that the crew's experience of infection by these organisms is similar to that of the general population. In view of the infrequent use of antibiotics during the patrols studied, the finding of an apparent increase in the number of resistant strains of *Staph. aureus* is interesting, especially as a similar trend was seen with respect to *N. meningitidis* isolated from nasopharyngeal swabs.

Despite the apparent dangers due to the presence of organisms of bowel origin in air samples and throat and nasal swabs of the crews, there is little evidence of illness as a result. Few experiments appear to have been reported on respiratory tract infections by bowel pathogens although much work has been carried out on the transmission of aerosols carrying faecal organisms. Darlow & Bale (1959) and Newsom (1972) examined the microbial hazards associated with flushing lavatories but the submarine situation is not comparable because high pressure air is released from the sewage tank after thorough agitation of the contents. Darlow, Bale & Carter (1961) showed that the lethal dose of *Salmonella typhimurium* in mice was lower when they were infected by inhalation than by ingestion, but they were concerned with much larger doses of the infectious agent($\sim 5 \times 10^4$ orgs/dose) than slit sampler counts show to be possible in the air of RN submarines. On the other hand, if the highest impinger counts of Watkins *et al.* (1970) are correct, there is reason for concern.

A detailed report on the frequency of and reasons for sick bay attendance is being prepared for publication, but United States Navy and Royal Navy experience is that the sickness rate falls during patrols and is lower in each patrol than the preceding one by the same crew. There is a highly significant reduction in sick bay attendance for upper respiratory complaints after the first two weeks of patrol, and it is concluded that the presence of microbial contaminants at the levels observed do not represent a threat to the wellbeing of the crews.

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