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Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*

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Background. **Numerous studies evaluating the efficacy of conjugate pneumococcal vaccines are being conducted or planned throughout the world. Some of these studies are evaluating the effect of vaccine on nasopharyngeal (NP) carriage.**

Methods. **The World Health Organization established a Working Group comprised of representatives from these trials and other NP colonization experts to establish core, standardized methods for the study of pneumococcal NP colonization that could be used in these trials. The intent was to reduce or eliminate variability in key methods which themselves could contribute to variability of observed pneumococcal NP colonization. In this way variability of vaccine effects between trials on NP colonization could more easily be analyzed for population or vaccine differences without the confounding effect caused by differences in study methodology.**

Results. **This paper presents the evidence base supporting the need for standardized NP colonization study methods, the methods themselves (Core Consensus Methods), including collection techniques, culture media, equipment, serotyping, storage of specimens and transport of isolates agreed on by the Working Group as well as a discussion of research priorities.**

Conclusions. **The Core Consensus Methods provide a common methodology to conduct pneumococcal NP colonization studies with minimum interstudy method variability. The intention is to allow more meaningful comparisons of study results from conjugate pneumococcal vaccine trials.**

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BACKGROUND

Streptococcus pneumoniae (pneumococcus) is the leading bacterial cause of infection worldwide, ranging from common infections such as otitis media to life-threatening invasive infections such as sepsis, meningitis and pneumonia.[1–3] It has one of the largest public health and economic impacts of any bacterial infectious disease agent in both developing and industrialized countries. Approximately 2.6 million children <5 years of age die annually of pneumonia predominantly in the developing world; approximately one-half of these deaths are attributable to *S. pneumoniae* either solely or in conjunction with a viral respiratory infection, malnutrition or HIV infection.[4]

Pneumococci are a part of the normal microbial flora of the nose and pharynx, particularly among young children, and are easily transmitted, usually by droplet secretions, from person to person. Transmission of pneumococci is increased during the course of other respiratory infections when secretions, coughing and sneezing are increased. Numerous studies have documented that acquisition of pneumococcus in the nasopharynx occurs earlier in life and is most prevalent among children in developing countries and indigenous populations in some developed countries (Table 1). A smaller number of studies have explored the cumulative prevalence and dynamics of pneumococcal nasopharyngeal (NP) carriage as an infant ages through the first year of life (Fig. 1). The studies represented in Figure 1 demonstrate that there are two general patterns of pneumococcal NP colonization: settings where children universally acquire pneumococcus in the first few months of life; and settings where the time of first colonization with pneumococcus is more commonly in the second 6 months of life or beyond. Only a few studies have systematically explored the relationship between the acquisition of carriage and progression to invasive disease or otitis media[7, 47] or the transmission of pneumococci within households and communities.[5, 9, 27, 48] Although these studies are few in number and represent specific geographic and socioeconomic settings, they have consistently shown that the pneumococcus is easily transmitted within families, usually

TABLE 1. Cross-sectional prevalence of pneumococcal nasopharyngeal carriage in children <24 months

Population (Ref)	Year	Subjects	Collection	Sample	Age in mo. (0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24)
Charlottesville, VA (5)	1975	18	Single swab	Community	38
Chapel Hill, NC (6)	1975	1229	Multiple	Day care	48 | 52
Birmingham, AL (7)	1980	82	Monthly (1-6), ever	Community	60
Australian Aborigines (8)	1985	174	Single swab	Community	89 | (14y)
Papua New Guinea (9)	1986	25	1- or 2-Weekly	Community	84 | 96 | 100
Johannesburg (10)	1986	254	Single swab	Day care	44 | (5y)
S Africa (Soweto) (11)	1986	303	Single swab	Day care	68 | (5y)
S Africa (Transvaal) (11)		156	Single swab	Day care	62 | (5y)
Australia (Adelaide) (12)	1988	1267	Single swab	Community	29 | (5y)
Zambia (13)	1988	150	Single swab	Community	21 | 17
Papua New Guinea (14)	1989	165	Single swab	Community	98 | (5y)
Papua New Guinea (15)	1990	155	Multiple	Community	100
Costa Rica (16)	1988-92	440	Multiple	Community	6 | 20 | 39 | 39
Sweden (17)	1992	468	2,6,10, 18 mo.	Well-baby clinics	12 | 30 | 32 | 32
Uruguay (18)	1992	373	Single swab	Clinics/ARI	42 | 56 | 39 | (5y)
		257	Single swab	Clinics/Healthy	12 | 14 | 24 | (5y)

TABLE 1—*Continued*

from an older sibling to a younger sibling and between households within communities. They have also shown that invasive disease is most likely to occur soon after nasopharyngeal colonization with a newly acquired serotype rather than after long duration of carriage of that serotype.[7, 47]

Pneumococcal disease and carriage are less frequent among adults than among children; however, disease rates increase again among the very elderly. Most attribute the lower risk of disease in the adult years to the acquisition of immunity, specifically antibodies to the capsular polysaccharide of the bacteria. The absolute concentrations of systemic antibody needed to protect against invasive disease are still undefined and may vary by serotype. Serotype-specific antibodies to the pneumococcal capsular polysaccharide are formed in response to carriage of *S. pneumoniae,*[49, 50] which provides a biologic basis for the observation that serotype-specific duration of carriage shortens with age.[7] However, children, particularly those <2 years of age, are much less likely to develop an immune response to carriage.[51, 52] Antibodies to common proteins of the pneumococcus such as pneumococcal surface adhesin A (PsaA) and pneumococcal surface protein A (PspA) also contribute to protection against carriage and disease, although these have been less well-studied.

Young children do not respond with an adequate antibody response to many pure polysaccharide antigens[53–55]; hence the currently licensed and available 23-valent pneumococcal polysaccharide vaccine has not been used as a tool for disease prevention in this age group. By conjugating polysaccharide antigens to a protein carrier, the antigen is converted from a T cell-independent one to a T cell-dependent one. Infants and toddlers can develop a brisk antibody response to the latter type of antigens. This strategy was successfully used to develop vaccines against *Haemophilus influenzae* type b (Hib). The protein conjugate vaccines against Hib are highly efficacious against invasive Hib disease and also induce indirect protection by preventing the acquisition of Hib naso/oropharyngeal carriage.[56–58] A similar strategy of conjugating the pneumococcal polysaccharide antigens to protein carriers has resulted in several pneumococcal protein conjugate vaccines that are licensed for use or in prelicensure clinical trials. One such vaccine, Prevnar (Wyeth, Pearl River, NY), is efficacious against invasive pneumococcal disease caused by serotypes included in the vac-

TABLE 1–*Continued*

Population (Ref)	Year	Subjects	Collection	Sample	Age in mo.
Pakistan (19)	1993	601	Single swab	Urban/ARI	64
		133	Single swab	Urban/Healthy	52
		285	Single swab	Rural/Both	61
Philippines (20)	1994	227	Single swab	Clinics/ARI	51 (5y)
Australian Aborigines (21)	1994	50	Multiple swabs	Community	>90
Finland (22)	1994-7	329	Multiple swabs	Community	9, 17, 22, 37, 43
Europe/Multicenter (23)	1996	954	Single swab	Healthy and Hosp	27 (5y)
Memphis, TN (24)	1996	216	Single swab	Well-baby clinics	47 (6y)
Israel (25)	1996	162	Multiple	Clinics	26, 32, 39, 34, 38, 43, 49, 46, 62
Alaska Natives (26)	1996	185	Single swab	Community	50 (5y)
The Gambia (27)	1996	1071	Single swab	Clinics/Clin Diag	85 (5y)
		81	Single swab	Clinics/IPD	90 (5y)
		113	Single swab	Community Ctrls	76 (5y)
Zambia (28)	1997	193	Single swab	Clinics	76
Malawi (29)	1997	200	Single swab	Clinics	34, 51, 51, 58, 47 (5y)
Greece (30)	1997	338	Single swab	Day care	39 (6y)
Mexico (31)	1997	406	Single swab	Clinics	60, 26
Buffalo, NY (32)	1997	306	1-6,8,10,12 mo.	Clinics	38, 54

TABLE 1–*Continued*

cine.[59] It has been licensed in the United States (February 17, 2000) for use among children through 9 years of age and recommended for routine use among children <2 years of age.[60] By the end of 2001 the vaccine also was licensed in Canada and most countries in Latin America and Europe.

Several reports now have documented the effect of conjugate pneumococcal vaccine on carriage of vaccine serotype and nonvaccine serotype (NVT) pneumococci. Regardless of vaccine product, age of administration or schedule, all studies have shown a reduction in carriage of vaccine serotype pneumococci among those immunized with pneumococcal protein conjugate vaccines.[61–67] These nasopharyngeal colonization studies have been conducted in various settings and populations around the world including Israel, The Gambia, American Indians, South Africa and Iceland. Several of these studies have also shown that carriage of NVT pneumococci has increased among the immunized children, suggesting that either replacement or unmasking of previously unidentified nonvaccine serotypes has occurred.[62–65, 67] Numerous studies have shown that children and adults may carry more than one serotype of pneumococcus at a time (Table 2).

The true prevalence of this phenomenon is still unknown. Pneumococcal carriage studies conducted early in the 20th century used laboratory techniques, such as mouse inoculation assays, with high sensitivity for detecting carriage of multiple serotype.[41] These assays are very labor-intensive, expensive and not practical for use in large NP studies. Thus most studies have relied on the technique of picking multiple pneumococcal colonies from a plate for serotyping. This strategy is an insensitive method for detecting multiple serotype carriage.[41]

Deciding how, when and where to use pneumococcal conjugate vaccines is dependent in part on a detailed understanding of their effect on NP carriage. Many questions remain unanswered and include among others the following. Can protection against pneumococcal carriage be used as a surrogate for vaccine efficacy? Will the pneumococci in the nasopharynx of vaccinated children be replaced by pneumococci with nonvaccine serotypes? If an increase in the prevalence of nonvaccine serotype pneumococci is observed in nasopharyngeal specimens from vaccinated children compared with children who have not been vaccinated, does this represent true replacement carriage? Or may these

TABLE 1–*Continued*

Population (Ref)	Year	Subjects	Collection	Sample	Age in mo.
Israel (33)	1998	120	Single swab	Clinics/AOM pts	63 (3y)
Gaborone, Botswana (34)	1998	130	Single swab	Well-child clinics	69
Francistown, Botswana (34)		44	Single swab	Well-child clinics	84
Toronto, Canada (35)	1998	532	Single swab	Day care	46 (4y)
Santiago, Chile (36)	1998	97	Single swab	Day care	59
Tamuco, Chile (36)		35	Single swab	Day care	14
Turkey (37)	1998	109	Single swab	Comm/Day-care	30
South India (38)	1999	100	6,10,14,18,22 wks,	Well-baby clinics	13 31 48 58 65 75 81
Toronto, Canada (39)	1999	423	Single swab	Day care	57
Portugal (40)	1999	586	Single swab	Day-care center	45 73
Israeli Jews *	2000	61	2, 7, 12, 13	Clinic	16 25 49 45
Israeli Bedouins *	2000	48	2, 7, 12, 13	Clinic	72 71 84 79
South Africa (41)	2000	303	Single swab	Clinics	40 (5y)
Greece (42)	2000	2448	Single swab	Clinics	31
Vietnam (43)	2000	389	Single swab	Community	50 (5y)
Central Africa Rep (44)	2000	371	Single swab	Clinics	73 (5y)
Indonesia (45)	2001	484	Single swab	Community	41 47 52
South India (46)	2001	464	2,4,6 mo.	Community	54 64 70

* Unpublished data.
ARI, acute respiratory infection; IPD, invasive pneumococcal disease; AOM, acute otitis media.

findings instead be the result of an unmasking of pneumococci with serotypes that were already present in the nasopharynx, but in lower numbers than pneumococci with vaccine serotypes, and therefore not detected before? What impact will changes in the serotype distribution of the carried pneumococci from vaccine types to nonvaccine types have on morbidity? What impact will changes in pneumococcal serotypes from vaccine to nonvaccine serotypes have on the carriage and spread of antimicrobial resistance pneumococci? Can we use carriage studies to predict the impact of vaccine use in different epidemiologic settings? Can the systemic or local immune response to vaccines predict the effect of these vaccines on carriage?

To compare the effect of various pneumococcal conjugate vaccines on NP colonization in different populations and geographic settings, an effort must be made to assure that observed differences in effect are not simply a result of variations in specimen collection techniques or laboratory methodologies. The anatomic site of sampling for culture, the method of sampling, the use of transport media, the culture media used, the laboratory conditions for growth and the techniques for isolation of pneumococci and identifying their type characteristics are all important determinants of the outcome of pneumococcal carriage studies. It has been noted previously that valid comparisons between studies cannot be made if different methods have been used for the isolation and identification of pneumococci.[73] As a result the World Health Organization, Department of Vaccines and Biologicals convened an ad hoc working group of investigators from the various ongoing or planned pneumococcal conjugate vaccine studies which include the effect on NP colonization as an outcome. The ad hoc working group met on April 2 to 3, 1998 and again on March 25, 2001; the main result of these meetings was the development of a consensus on core, standardized methods for NP studies to allow comparison of results between studies. These methods are presented below.

CORE CONSENSUS METHODS

The following was developed as the standard methodology for detecting nasopharyngeal pneumococcal colonization in studies conducted within the context of

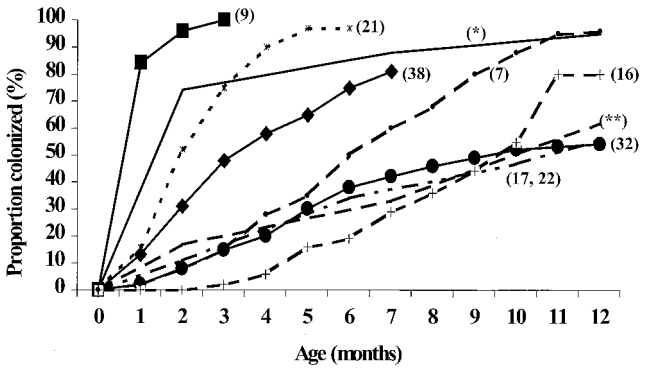

FIG. 1. Cumulative prevalence of pneumococcal NP colonization by age in various populations. Numbers in parentheses, references. *, Israeli Bedouins; **, Israeli Jews (R Dagan, unpublished data).

pneumococcal conjugate vaccine trials. This methodology also could be used as a standard for other studies of pneumococcal carriage in nasopharynx.

1. Sample collection. Nasopharyngeal samples are obtained with a deep nasopharyngeal swab. To obtain the specimen the patient's head should be tipped slightly backward and the swab passed directly backwards, parallel to the floor of the nasopharynx. The swab should pass without resistance until it reaches the posterior pharynx which is approximately one-half to two-thirds the distance from the nostril to the ear lobe. If resistance is encountered, the swab should be removed, and an attempt should be made to pass the swab through the other nostril. Once the swab is in place, rotate the swab 180 degrees or leave it in place for 5 s to saturate the tip before removing it slowly. Often failure to obtain a good specimen results from the failure to pass the swab fully into the posterior nasopharynx. A record should be kept of the presence or absence of nasal mucus and the success of the procedure as acceptable or suboptimal.

2. Swab. Swabs should be pediatric size with a calcium alginate or Dacron polyester tip and a flexible aluminum shaft. Studies in the 1930s and 1940s indicated that cotton swabs had properties inhibitory to pneumococcus.[74, 75] These authors speculated that the fatty acids contained therein were inhibiting the growth of the organism. We could identify no reports on whether cotton swabs produced in the current era also suffer from this problem. As a result, we recommend the use of calcium alginate or Dacron polyester swabs. For any single study the type and supplier of the swab should be consistent whenever possible.

3. Swab transport media. Transport and storage media have been used by some investigators to store original NP material for long periods of time.[76] Recently the most commonly used medium, skim milk-tryptone-glucose-glycerin (STGG), has been validated against the standard method of direct inoculation of NP swabs onto culture plates.[77] The STGG medium is as good as or better than direct inoculation for recovering pneumococci. The methodology is as follows.

Once a swab specimen is collected, it is placed in a tube of STGG transport medium. Typically a calcium alginate swab is immersed and stored in 1 ml of STGG medium, but other volumes may be used. The STGG medium has several advantages over direct plating. These include long term storage of the original NP

TABLE 2. Proportion of children with multiple serotype pneumococcal carriage in the upper respiratory tract

Year	Population	No. of Subjects	Carriage Rate (%)	No. of Pnc Positive	Multiple Types Detection Method	No. Colonized with >1 Serotypes		
						2 types	3 types	≥4 types
1933[68]	German	95	95	NA	MI	54 (57)*	14 (15)	2 (2.1)
1944–1945[69]	US Air Force	NA	59	1549	MI	200 (12.9)	27 (1.7)	5 (0.4)
1965–1969[48]	New York	NA	4.6	890	NA	12 (1.3)		
1974–1975[7]	Alabama	82	61.2	573	NA	37 (6.5)	3 (0.5)	0
1980–1982[70]	PNG	NA	~100	156	≤6 colonies	43 (27.6)	3 (1.9)	0
1980–1981[12]	Australia	269	20	269	Multiple colonies	89 (33)	6 (2)	2 (1)
1981[8]	Australian Aborigines	282	68.4	25	4 colonies	2 (12)		
				209	Multiple colonies	15 (5)		
1981[14]	PNG	NA	98	161	Multiple colonies	42 (26.1)	2 (1.2)	0
1982–1983†	PNG	NA	NA	139	NA	30 (21.6)	3 (2.1)	0
1984†	PNG	NA	NA	10	50 colonies	4 (40)	1 (10)	0
1985–1987[15]	PNG	158	100	1449	NA	427 (29)	48 (3)	3 (0.2)
1989–1991[27]	Gambia	1071	85.1	911	LAG	105 (11.5)		
1990–1991[71]	Australian Aborigines	NA	NA	136	Multiple colonies	28 (20.6)	1 (0.7)	0
1996[26]	Alaska Natives	185	50	92	Multiple colonies	3 (3.3)	0	0
1998‡	Gambia	200	94	188	LAG	19 (10)	6 (3)	0
1999[72]	Spain	332	36.1	120	Multiple colonies	8 (6.7)	0	0
2000[41]	South Africa and Israel	NA	NA	1899	3–5 colonies	28 (1.5)	0	0

* Numbers in parentheses, percent colonized.
† M. Gratten, M.Sc. thesis, 1984, personal communication.
‡ R. Adegbola, personal communication.
MI, mouse inoculation study; LAG, latex agglutination of pneumococcal culture; NOS, not otherwise specified; NA, not available in publication; PNG, Papua New Guinea.

specimen, the opportunity to inoculate multiple plates from the original sample, homogenous dispersion of the NP specimen and the ability to quantitate the growth of organisms. The medium is not commercially available; therefore production and quality control must be conducted locally. Production of STGG transport medium is based on the formula described by Gibson and Khoury.[78]

Mix together the following products (The manufacturer and lot numbers of ingredients should be standardized for a given study whenever possible.): Oxoid tryptone-soya broth (CM 129) 3.0 ml; glucose 0.5 g; Oxoid skim milk powder (CM L31) 2.0 g; glycerol 10.0 ml; double distilled water 100.0 ml. Dispense in 0.5- to 1.0-ml amounts in Nunc or Nalgene cryotubes, and autoclave at 15 lb for no more than 10 min. Tighten the lids before storage. The tubes may then be stored refrigerated (4–6°C) or at room temperature (22°C) for several months. Before use the pellet in the bottom of the tube should be resuspended by vortexing for at least 10 to 15 s.

Quality control of STGG transport medium should be conducted to assure sterility and the ability of the medium to sustain pneumococci. Some suggested quality control procedures are detailed here. The solution should be tan with a precipitate at the bottom of the tube. Select at random one tube from each cryobox in each lot. Vortex until the precipitate is suspended, and plate 100 μl onto a blood agar plate. Incubate overnight at 37°C in 5% CO_2 and examine the plate for any growth. There should be no growth whatsoever. Vortex a tube to suspend the precipitate, and inoculate it with a representative strain of pneumococcus. Freeze at −70°C for 48 h. Thaw out the specimen at room temperature, vortex again, subculture 100 μl onto a blood agar plate, streak and incubate overnight at 37°C in 5% CO_2. The organism should show good growth.

4. Specimen transport and storage procedures. Once the NP specimen is collected, place the NP swab in a labeled vial with 0.5 to 1.0 ml of STGG transport medium. Using scissors sterilized with an alcohol wipe, aseptically cut off the excess wire handle from the swab, leaving the swab itself in the transport medium, and tighten the cap. Maintain and transport the specimen on wet ice to the local laboratory within 8 h. In the laboratory vortex the specimen for 10 to 20 s to disperse organisms from the swab tip, and freeze with the swab in the medium at −70°C. The specimen may also be maintained refrigerated for <5 days, or frozen at −20°C for no longer than 6 weeks. The specimen may be separated into two aliquots; however, because the swab is maintained in only one of the aliquots they may not be identical over time.

5. Culture techniques. Columbia or Trypticase soy agar-blood agar (BA) with blood from horse, sheep or goat is acceptable. Human blood should not be used. Unless the plates have already been tested for their ability to support growth of pneumococci, one plate from each lot should be tested before using that lot of plates. Selective BA plates with 2.5 μg/ml or 5.0 μg/ml gentamicin, colistin-nalidixic acid or colistin-oxolinic acid are considered as part of the core method. The decision to use nonselective BA plates should be based on the need or lack thereof to suppress growth of other organisms carried in the population under study.

The primary culture should be obtained either before the specimen is frozen or by fully thawing a frozen STGG specimen at room temperature. The specimen should be mixed thoroughly using a vortex and a predetermined volume (e.g. 10 or 50 μl)of the vortexed specimen from the STGG tube (i.e. with a loop or a pipet) is inoculated onto a selective plate and incubated at 35–37°C in 3 to 10% CO_2 overnight. Record the semiquantitative growth of alpha-hemolytic colonies. After inoculating the specimen in Quadrant 1 of a plate and streaking into all four quadrants, the semiquantitative measurements can be done in various ways. One such method is assessed as 4+ if >10 colonies are in Quadrant 4, 3+ if there are <10 colonies in Quadrant 4 and >10 colonies in Quadrant 3, 2+ if there are <10 colonies in Quadrant 3 and >10 in Quadrant 2 and 1+ if there are <10 in Quadrant 2 but growth in Quadrant 1.

From the primary plate pick and streak out on one half of a BA plate two presumptive pneumococcal colonies, attempting to select colonies that look as different as possible; place an optochin disc in the center of each streak, and incubate overnight as above. Susceptibility to optochin is defined as the diameter of inhibition zone and depends on the disc size, the disc manufacturer and the methods of incubation.[79] Zones of inhibition >14 mm indicate susceptibility, 7 to 13 mm are indeterminate and a zone <7 mm is resistant to optochin when a 6-mm size disc is used and the culture is incubated in 5% CO_2. Isolates that are optochin-susceptible are considered pneumococci; those of intermediate susceptibility should be tested for bile solubility. Those that are optochin-resistant are considered to be a species other than pneumococcus; however, a small number of pneumococci may be optochin-resistant. Serotype and store pneumococcal strains isolated in this manner (see Paragraphs 7 and 8).

6. Storage of original NP sample. Novel, sensitive methods for detection of multiple pneumococcal serotypes from the original NP specimen are being developed; therefore the remainder of the NP specimen should be stored in STGG medium at −70°C for future use. NP specimens can be preserved in this fashion for extended periods of time. Recent studies have demonstrated comparable qualitative and semiquantitative recovery of pneumococci 2 and 6 years after collec-

tion.[76] If the NP specimen cannot be stored, a second option is to save a sweep of the growth from the primary plate into STGG medium or into 15% glucose-glycerol stored at −70°C.

7. Storage of pneumococcal isolates. From the optochin plate pick one or more isolated pneumococcal colonies and streak them as a lawn onto a BA plate. After overnight incubation examine the growth for purity. If the growth is pure, harvest it with a sterile swab and dispense the growth into two tubes of STGG medium. Freeze each suspension at −70°C. If the growth is not pure restreak a BA plate from one colony to obtain a lawn of pure growth. The isolates in STGG can, if necessary, be also stored at −20°C for at least 12 months (T Kaijalainen and E Herva, personal communication). To recover the isolate remove a tube from the freezer and place on dry or wet ice depending on the conditions under which the isolate was frozen. In a laminar flow cabinet, remove the screw cap and, using a loop, inoculate a small amount of frozen material into broth or onto solid medium. Return the tube to the −70°C freezer immediately or maintain the specimen on dry ice before any thawing has occurred.

8. Serotyping pneumococcal isolates. The Neufeld (Quellung) reaction is considered to be the standard method for serotyping of pneumococci. Serotyping by the Quellung reaction with the use of type-specific pneumococcal rabbit antisera can be done from the pure culture on BA either obtained directly after isolation or recovered from a frozen culture. Some laboratories prefer other methods such as counterimmunoelectrophoresis, latex agglutination or flow cytometry, after standardizing these methods against the Quellung reaction. Currently efforts are being made to evaluate the serotyping procedures between laboratories around the world through a European Union funded project (HB Konradsen, personal communication).

9. Transport of pneumococcal strains. Strains of pneumococcus may be transported by a variety of methods. Recently Dorset egg medium has been shown to be a convenient and effective means for transporting strains.[80] It appears to be superior to Columbia agar base medium[81] for long term preservation of specimens. Dorset egg medium should be inoculated rather than stabbed with the isolate.

10. Serotyping from the original NP sample. No validated method is currently available to serotype pneumococci or to identify simultaneous carriage of multiple serotypes from the original NP sample without a culture step. For this reason it is recommended that the original NP sample be maintained in STGG medium at −70°C until appropriate methods are developed.

COMMENT

S. pneumoniae belongs to the normal upper respiratory flora of humans. There may be evolutionary reasons why human infants do not develop antibodies to many polysaccharide antigens. Although much has been characterized, many details of pneumococcal pathogenesis, host-pathogen interaction, dynamics of carriage in families and communities and transition from carriage state to overt disease still remain unknown.

There remain major constraints to full understanding of the ecology of pneumococcus in the nasopharynx. Of primary importance is the ability to detect simultaneous carriage of multiple pneumococci with different serotypes in the original NP samples. At the same time it would be a major advantage to be able to quantify the relative proportion of the different serotypes present in the specimen. At present all methods rely on a culture step before identification of serotypes. In addition standard methods that sample multiple colonies have limited sensitivity for detecting minor populations of second or more serotypes.[41] Recent advances to improve the methodology for detection of multiple serotypes from a cultured specimen include the development of a new immunoblot method[82] and a serum broth enrichment method.[83, 84] The former method, although capable of detecting a minority population occurring at a rate of only 1% of the majority population is time-consuming and very labor-intensive. The serum broth enrichment method is based on the use of serum broth as both a transport and a culture medium. After incubation of the transport medium, serotyping is performed directly on the transport medium. A culture step may modify the relative proportions of the different pneumococcal populations in the specimen; as well it is theoretically possible that culturing can change the character and/or expression of the capsule.

The goal is to develop methods to detect pneumococcal serotype directly from NP material without a culture step; various investigators recently have attempted to develop such methods. Preliminary studies developing a quantitative PCR-based method have been promising (M Leinonen, personal communication). This method is appealing because the relative proportions of the present serotypes can be quantified and because PCR technology is available at most research centers. The method, however, relies on known primer sequences capable of recognizing sequences on the pneumococcal chromosome coding for capsular genes. Others have explored the use of latex agglutination, flow cytometry and immunoblot methods, all with relatively disappointing results in terms of sensitivity of the assay.

A promising strategy, and to our knowledge one not yet explored for this purpose, is the use of new DNA microarray technology, a method theoretically capable

of screening a large number of specimens for the presence of multiple serotypes. Both of the techniques, PCR and DNA microarray technology, are dependent on sequence data that characterize the capsular genes for important pneumococcal serotypes, and ideally for all known serotypes. Therefore there is an urgent need for an initiative to sequence the capsular genes of the pneumococcal serotypes not already sequenced. Equipped with these new molecular biologic techniques, one should be able to provide answers to some of the major questions concerning pneumococcal carriage, such as simultaneous carriage of multiple serotypes and carriage dynamics. It will also be possible to screen NP specimens for genes encoding antimicrobial resistance and pathogenic factors other than the capsule. Even though these new promising technologies have a lot of advantages, it must be remembered that these methods are limited to finding what they are constructed to seek, and do not render cultured pneumococcal isolates obsolete. Isolates are needed for molecular epidemiologic typing, for testing sensitivity to antibiotics, for detecting previously unknown serotypes and for identifying new mechanisms of antimicrobial resistance in pneumococci.

In addition to methods to detect carriage of multiple serotypes, there is a need to reach a consensus on methods for epidemiologic typing of the carried isolates. Epidemiologic typing is essential for answering questions about carriage dynamics and mechanisms of pneumococcal transmission as well as for surveillance of pneumococcal disease. Capsular serotype, antimicrobial susceptibility pattern and multilocus enzyme electrophoresis have been supplemented by new molecular epidemiologic typing methods such as ribotyping, pulsed field gel electrophoresis, ox PCR and multilocus sequence typing. Consensus on a common nomenclature for the characterized pneumococcal clones is needed to allow for comparisons between studies, making observed changes in the epidemiology of the pneumococci resulting from antibiotic pressures and vaccine introduction a function of the interventions rather than the method of analysis.

The data collected in carriage studies usually include multiple repeated observations over long time periods with multiple dimensions of interaction. Recently mathematical sciences have developed new theories and modeling techniques that can provide tools for the carriage dynamics.[85–87] With the development of modern analytic techniques, it is now possible to build more realistic models to predict changes in the pneumococcal flora.[88]

This standardized protocol for detection of NP carriage of pneumococci and the development of new, more sensitive means of detecting multiple serotypes in the same specimen will be the tools that can forward our understanding of the ecology of *S. pneumoniae* in the nasopharynx of humans and the communities they constitute. This understanding will advance evaluating the impact of new pneumococcal vaccines on this ecology.

The future research needs in the area of pneumococcal nasopharyngeal colonization and disease are many. Some of the key research areas related to the evaluation and introduction of polysaccharide protein conjugate pneumococcal vaccines include the following.

1. Establish long term surveillance for pneumococcal NP carriage in areas where conjugate vaccine is being introduced. Preventing carriage of the most common, vaccine-specific serotypes of *S. pneumoniae* from the normal flora of the human host may not occur without adverse effects. Therefore large scale pneumococcal conjugate vaccine trials and postmarketing surveillance studies should attempt to evaluate the effect of vaccine on both pneumococcal invasive disease and NP carriage. It is important to extend these studies to the close contacts of the vaccinated infants as well as their community contacts in order to understand the indirect effects of the vaccine as well (e.g. herd immunity). Surveillance of pneumococcal isolates causing invasive disease must be paired with surveillance of pneumococcal isolates from noninvasive disease (e.g. acute otitis media) and carried isolates. Shifts in the serotype distribution of carried pneumococci may or may not result in changes in the distribution of serotypes among invasive disease or noninvasive disease causing organisms. An increase in NVT carriage among Finnish infants vaccinated with either of two seven valent conjugate pneumococcal vaccine products did result in an increase in otitis media from nonvaccine type pneumococci.[89, 90] These are the only reports of an increase in disease from NVT strains. Further data are needed to determine the extent of the otitis media replacement phenomenon, focusing not only on nonvaccine type pneumococci, but also other species causing otitis media and disease severity trends.

2. Identify a method to transport NP samples that requires less stringent cold chain requirements than the STGG medium. There are many areas around the world where the requirements set out in this protocol cannot be met. There is a need, therefore, to develop, identify and assess other methods for transporting NP samples in ways that will preserve the pneumococci under the heat and humidity conditions that are commonly encountered.

3. Develop a method to store NP samples that minimizes storage space requirements. Although the STGG method is practical from many perspectives, when thousands of samples are being collected, issues of storage space arise. To preserve the large specimen banks that are being developed in the context of pneumococcal NP studies, more space-efficient storage methods of the NP specimens should be developed.

4. Develop methods to detect multiple serotype carriage directly from NP samples. As discussed previously methods for identifying the pneumococci present in NP specimens are critical to assessing the impact of vaccines on the ecology of the pneumococcus. There is a pressing need to characterize the genes that encode all pneumococcal serotypes, beginning first with those in the various candidate vaccines, followed by those nonvaccine serotypes that are most the prevalent causes of invasive disease, noninvasive disease and colonization.

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APPENDIX

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