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Generic protocol to estimate the burden of *Shigella* diarrhoea and dysenteric mortality

Field test version, May 1999

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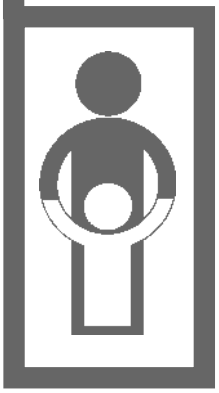

DEPARTMENT OF VACCINES AND BIOLOGICALS

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Preface

The WHO Department of Vaccines and Biologicals (V&B) has an increasing interest in vaccines against *Shigella*, and several candidate vaccines are being tested in clinical trials. The promise of having a new generation of vaccines available in the relatively near future emphasizes the need to understand the disease burden and the epidemiology of *Shigella* infection in developing countries.

The V&B Steering Committee on Epidemiology and Field Research and the V&B Steering Committee on Diarrhoeal Disease Vaccines jointly identified the need for a practical method for immunization programme managers and clinical epidemiologists to assess the local disease burden due to *Shigella*. At the request of these Steering Committees, this generic protocol was prepared by staff at the U.S National Institute of Child Health and Human Development, the University of Maryland, and the U.S. Centers for Disease Control and Prevention.

This protocol provides a general outline for a study and describes the main procedures involved. However, it will need to be adapted to the local setting, and details of field work and operational procedures should be added by local investigators with experience in conducting field studies of diarrhoeal diseases.

WHO provides this protocol free-of-charge. In return, WHO would appreciate being informed about studies conducted using this protocol. This WHO document should be referenced in any publication resulting from its use.

Comments or suggestions for improving this generic protocol are welcome and should be sent to the Department of Vaccines and Biologicals, WHO, 1211 Geneva 27, Switzerland.

1. Introduction

1.1 The global burden of *Shigella*

Several ongoing trends underscore the limitations of modern medical and public health efforts in controlling shigellosis in developing countries and emphasize the need for a safe and effective *Shigella* vaccine. For one, successful implementation of oral rehydration programmes to treat watery diarrhoea in developing countries has increased the relative importance of dysentery and persistent diarrhoea as clinical problems [1]. At a diarrhoeal disease centre in Bangladesh between 1975 and 1985, for example, deaths among children 1 to 4 years old attributed to acute or chronic dysentery outnumbered deaths attributed to acute or chronic watery diarrhoea by a factor ranging from 2.1 to 7.8 [2].

Another concern is the growing problem of antimicrobial resistance. Over the years, sulfonamides, tetracycline, ampicillin and trimethoprim/sulfamethoxazole initially appeared as highly efficacious drugs, only to become impotent in the face of emerging resistance [3]. In the 1990s, few reliable options exist to treat multi-resistant *Shigella* infections, particularly in developing countries where cost and practicality are important considerations.

A third problem is the ease with which person-to-person transmission occurs, presumably the result of the small infectious inoculum [4]. One serotype, designated *Shiga* (*S. dysenteriae* type 1), is capable of spread in an epidemic and pandemic pattern, often affecting populations in areas of political upheaval and natural disaster [5]. An example of the devastation that can be produced by *Shiga* occurred among the 500 000-800 000 Rwandan refugees who fled into the North Kivu region of Zaire in 1994. During the first month alone, approximately 20 000 persons died from dysentery caused by a strain of *S. dysenteriae* type 1 that was resistant to all of the commonly used antibiotics [6].

In many regions of the developing world, the HIV epidemic intersects with spread of shigellosis. HIV-associated immunodeficiency leads to more severe clinical manifestations of *Shigella* infection, including persistent or recurrent intestinal disease and bacteremia [7-9].

Advances in biotechnology have enabled the development of a new generation of candidate vaccines. The state of progress in the development and testing of *Shigella* vaccines was recently reviewed at a meeting convened by the World Health Organization [10]. Some of these vaccines have entered clinical trials and show great promise for the prevention of *Shigella* disease [11-13].

The purpose of this generic protocol is to assist studies in developing countries that will allow estimation of the local disease burden of *Shigella* infection. Information will be generated to guide the choice of relevant *Shigella* serogroups and serotypes for inclusion in a vaccine, and to identify potential sites for field trials. The results are also intended to assist public health officials when they consider cost effectiveness, target populations, and other issues necessary for introducing *Shigella* vaccines to their immunization programme and for evaluating the impact of these vaccines that have been introduced [14].

1.2 The organism

Shigellae are gram negative, non-lactose fermenting, nonmotile bacilli of the family *Enterobacteriaceae*. They are classified into four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*, also designated Groups A, B, C and D, respectively. Groups A, B, and C contain multiple serotypes (13, 6 (with 15 subtypes) and 18, respectively), whereas Group D contains only a single serotype.

1.3 Pathogenesis of *Shigella* infections

After oral inoculation, *Shigellae* pass to the terminal ileum and colon where they invade and proliferate within epithelial cells, spreading cell-to-cell. This process evokes an intense local inflammatory response, with formation of mucosal microabscesses and ulcerations. Two *Shigella* enterotoxins, designated ShET1 and ShET2, have been incriminated as the putative mediators of the watery diarrhoea seen early in the clinical course [15,16]. Although all *Shigella* species elaborate varying quantities of exotoxins, only *S. dysenteriae* 1 produces the highly potent cytotoxin called Shiga toxin. Shiga toxin production is not required for virulence of invasive *S. dysenteriae* strains, but may increase the severity of disease by destroying the endothelium in the local capillary loops and causing ischemia in the intestinal tissue. In addition, Shiga toxin is implicated as the etiologic agent of the thrombotic microangiopathy that characterizes hemolytic uremic syndrome sometimes seen following infection with *S. dysenteriae*.

1.4 Clinical findings of *Shigella* infections

Shigellosis typically evolves through several phases. The incubation period is 1 to 4 days, but may be as long as 8 days with *S. dysenteriae* [17]. The first symptoms to occur are fever and other constitutional symptoms such as headache, malaise, anorexia, and occasional vomiting. Watery diarrhoea typically precedes dysentery [18] and is often the sole clinical manifestation of mild infection [19]. Progression to frank dysentery may occur within hours to days with frequent small stools containing blood and mucus, accompanied by lower abdominal cramps and rectal tenesmus. Patients with severe infection may pass more than 20 dysenteric stools in one day [20]. With repeated infections by the same serotype, illness is absent or attenuated and excretion is diminished [21-23].

A variety of unusual extraintestinal manifestations may occur. The most common is seizures, which usually occur in the presence of fever without associated encephalopathy [24]. Microangiopathic hemolytic anemia can complicate infection with Shiga toxin-producing organisms, manifesting as the hemolytic uremic syndrome in children and as thrombotic thrombocytopenic purpura in adults [25]. Rarely, *Shigella* disseminates to distant sites causing focal infections such as meningitis, arthritis, splenic abscess, and osteomyelitis.

Most episodes of shigellosis in otherwise healthy individuals are self-limited and resolve within 5 to 7 days without sequelae. Acute, life-threatening complications are most often seen in malnourished infants and young children living in developing countries [26]. These include metabolic derangements, such as dehydration, hyponatremia, and hypoglycemia [26], intestinal complications such as toxic megacolon, rectal prolapse, intestinal perforation [26], and rarely sepsis [27]. *Shigella* bacteremia has been reported among HIV-infected and other immunocompromised patients [8,9].

Persistent diarrhoea and malnutrition are the most common chronic sequelae [28]. A rare post-infectious complication seen primarily in adults following infection with *S. flexneri* serotypes is reactive inflammatory arthritis, alone [29] or as part of a constellation of arthritis, conjunctivitis, and urethritis known as Reiter's syndrome [30]. Individuals harboring the HLA-B27 histocompatibility antigen account for approximately one-half of the cases [31].

1.5 Immunity to *Shigella* infections

Several lines of evidence indicate that wild type *Shigella* infection confers protective immunity. In endemic areas, the incidence of shigellosis peaks during the first 5 years of life and declines thereafter, suggesting that immunity develops after repeated exposures during childhood [19]. The incidence of disease declines with the duration of stay in high-risk settings such as military camps [32]. Of great relevance to vaccine development is the observation that this immunity is serotype-specific (e.g., directed to the LPS O antigen of the organism). Compelling evidence of serotype-specific natural immunity comes from a longitudinal study of a cohort of Chilean children in whom primary *Shigella* infection conferred 76% protective efficacy against reinfection with the same serotype [21]. Moreover, adult volunteers who were experimentally infected with either *S. sonnei* or *S. flexneri* were significantly protected against illness following rechallenge with the homologous strain (64-74% protective efficacy) [22,23]. Because immunity to *Shigella* is serotype-specific, the protective performance of an anti-*Shigella* vaccine in any particularly setting will depend in part on the representation of serotypes in the vaccine and on the relative epidemiological importance of different serotypes in that setting. Thus, knowledge of the distribution of serotypes among clinical isolates is of crucial importance in designing new vaccines and in judging their suitability for use in public health programmes.

1.6 Diagnosis of *Shigella* diarrhoea

There is no commercially available serologic assay to aid in the diagnosis of shigellosis. Definitive diagnosis of *Shigella* infection is made by culturing the organism from a stool specimen. Areas of faecal mucus are optimal for sampling. If whole stool is not available, comparable culture results can be obtained by gently swabbing the rectal mucosa with a cottontip applicator inserted into the anus [33]. *Shigellae* are extremely fastidious and survive poorly in stool samples that are left in ambient temperature; therefore, the sample should be fresh. If it cannot be plated quickly onto solid media, the specimen should be inoculated into transport media and refrigerated. Buffered glycerol saline may be preferable to Cary Blair as a transport media [34].

A variety of mildly selective as well as highly selective media are appropriate for culturing *Shigella*. Yield of culture can be enhanced by the addition of enrichment media such as Gram negative (GN) broth. Antibiotic susceptibility testing should be performed. Many laboratories serogroup using commercial antisera but serotyping is usually delegated to reference laboratories.

1.7 Epidemiology of shigellosis

1.7.1 Reservoirs and modes of transmission

Humans are the only natural hosts for *Shigella*. Worldwide, the incidence of shigellosis is highest among children 1 to 4 years old. The predominant mode of transmission is by faecal-oral contact, and the low infectious inoculum (as few as 10 organisms)[4] renders *Shigellae* highly contagious. Persons symptomatic with diarrhoea are primarily responsible for transmission [35].

Less commonly, transmission is related to contaminated food and water or fomites; however, the organism generally survives poorly in the environment. In certain settings where disposal of human faeces is inadequate, houseflies can serve as a mechanical vector for transmission [36].

1.7.2 Burden of shigellosis *in developing countries*

Proper estimation of the burden of *Shigella* disease in developing countries has two important dimensions: a *clinical* dimension that provides the magnitude of morbidity and mortality attributable to this pathogen and a *biological* dimension that provides the distribution of *Shigella* serotypes in different settings.

1.7.2.1 The clinical dimension: morbidity and mortality

S. dysenteriae type 1, also known as Shiga bacillus, has been recognized as the major cause of epidemic dysentery for nearly 100 years. During the past 30 years, pandemics of Shiga dysentery have spread across Central America, Bangladesh, South Asia and Central and East Africa [5,37,38], particularly affecting populations in areas of political upheaval and natural disaster [5]. This devastating form of shigellosis is associated with high rates of illness (attack rates have ranged from 1.2% in El Salvador to 32.9% during an outbreak on St. Martins Island) and case-fatality (ranging from 0.6% during an epidemic in Burma to 7.4% in the Guatemalan epidemic) [2,37-41]. The pandemic that began in Central Africa in 1979 progressed to East Africa and

has since become particularly problematic among refugee populations [5]. The pandemic strains often exhibit multiple antibiotic resistances and induce severe illness with high case fatality in all age groups [2,42]. In general, both the incidence and the fatality rates are highest among the very young and the elderly. However, available data only permit an estimation of deaths that occur during the acute or subacute phase of shigellosis. Deaths that result after extended periods of persistent diarrhoea, intestinal protein loss, and chronic malnutrition following shigellosis could not be measured.

Although epidemic Shiga dysentery is the most dramatic manifestation of *Shigella* infection in developing countries, the majority of *Shigella* infections are due to endemic shigellosis. Endemic *Shigella* is responsible for approximately 10% of all diarrhoeal episodes among children younger than five years living in developing countries [21] and up to 75% of diarrhoeal deaths [2,43]. In Bangladesh alone, *Shigella* dysentery causes 75 000 deaths among children in this age group annually during peak epidemic years and an estimated 35 000 deaths in non-epidemic years [2].

A recent review of the literature [43] concluded that, of the estimated 165 million cases of *Shigella* diarrhoea that occur annually, 99% occur in developing countries, and in developing countries 69% of episodes occur in children under five years of age. Moreover, of the ca. 1.1 million deaths attributed to *Shigella* infections in developing countries, 60% of deaths occur in the under-five age group. This picture contrasts with that for the other main causes of infectious diarrhoea in developing countries— rotavirus and enterotoxigenic *Escherichia coli*— for which the overwhelming burden of morbidity and mortality occurs in children under three years of age.

Although generally instructive, these conclusions are limited by several features. First, although estimates of the burden are based on multiple studies for children, data are limited for adults, who may account for a substantial fraction of *Shigella* morbidity and mortality and who may thus constitute an important target group for future vaccines. Second, it is appreciated that in developing countries a substantial fraction of *Shigella* deaths occur in persons who never seek medical care or who die after discharge from the hospital. Indeed, it has been estimated that the absolute number of persons who die from *Shigella* infections out of hospital may outnumber the absolute number of *Shigella* deaths in the hospital by six-fold. Yet, estimates of out-of-hospital *Shigella* mortality are based on very few studies [2].

1.7.2.2 The biological dimension: distribution of serogroups and serotypes

The predominant serogroup of *Shigella* circulating in a community appears to be related to the level of socioeconomic development. Attempts have been made to summarize published studies which quantify the proportion of isolates from hospitalized patients, presumed to be the most severe cases, throughout the world, recognizing that considerable variation exists among studies [43]. *S. flexneri* is the main serogroup found in developing countries (median 60% of isolates), with *S. sonnei* being the next most common (median 15%) [43]. *S. dysenteriae* (which is seen most often in South Asia and sub-Saharan Africa) and *S. boydii* occur with equal frequency (median 6%) [43]. In contrast, data from Spain, Israel and the United States consistently demonstrate that *S. sonnei* is the most common serogroup found in industrialized countries (median 77%), followed by *S. flexneri* (median 16%), *S. boydii* (median 2%) and finally *S. dysenteriae* (median 1%) [43].

Because immunity to *Shigella* is serotype-specific and vaccine protection will therefore depend on the representation of serotypes in the vaccine, knowledge of the distribution of serotypes in addition to serogroups of clinical isolates is of crucial importance in designing new vaccines. The distribution of serotypes reported among patients who have been hospitalized with shigellosis has been reviewed [43]. Among *S. flexneri* isolated from developing countries, serotype 2a caused 32-58% of infections, followed by serotype 1b (12-33%), 3a (4-11%), and finally 4a (2-5%) and 6 (3-5%). Among *S. dysenteriae* isolates, type 1 predominated in Guatemala, India and Malaysia (median for developing countries 30%), while type 2 predominated in Yemen (median 10%). The remaining *S. dysenteriae* serotypes identified in developing countries were 4, 5, 6, 7, 9 and 10. The predominant *S. boydii* serotype was 14 in India, Nigeria and Yemen where it accounted for 23-47% of isolates. In Guatemala serotype 2 predominated (40%). The wide variations in these distributions together with the rather limited number of countries for which serotype distributions have been studied underscores the need for more studies on this topic.

1.8 The need for further studies of disease burden

As alluded to in the above comments, existing studies document that *Shigella* infections are a major source of morbidity and mortality in the developing world, but are insufficient for the purpose of guiding the development and introduction of new-generation vaccines against this group of enteropathogens.

First, although several hospital-based studies document the relative importance of shigellosis, there have been few studies with a defined population denominator that allowed calculation of incidence rates in the community. There is little information on the rate with which infected persons seek outpatient and inpatient medical care, or other similar measures of disease severity.

Second, it is well documented that *Shigella* sp. are fragile organisms that may be missed in routine microbiological evaluations of faecal specimens. Considerable care must be exercised in collecting faecal specimens, transmitting them to diagnostic laboratories, and in using appropriate media for isolation. The existing literature on shigellosis represents an admixture of studies that used optimal and suboptimal isolation techniques.

Further studies, done with optimal isolation techniques, are needed for accurate estimation of disease burden.

Third, antimicrobial resistance constitutes an important element of disease burden. Patterns of antibiotic resistance, which vary considerably from place to place and which are in a continuous state of evolution, must also be updated.

Fourth, it is thought that an appreciable fraction of shigellosis and *Shigella*-mortality occurs in persons over the age of five years, and that the burden is significant in adults. However, few studies have included adequate numbers of subjects in older age groups to determine the disease burden both in the community and in health care settings. This information is important when considering the target populations for immunization.

Fifth, there are more than 40 serologically distinct groups and types of *Shigella*; however, only a few studies done in the developing world have characterized the prevalence of serogroups and serotypes by region. Published studies have originated from Chile [21], Malaysia [44], Philippines [45] and Yemen [46]. Additional data are thus needed from all areas of the world, and in particular sub-Saharan Africa and South Asia. Since most of the vaccines under development are designed to confer serotype-specific immunity, this information is necessary to guide vaccine development and implementation.

Finally, mortality from shigellosis is a critical issue in considering its disease burden, but available information is derived from a limited number of sites. Estimates of case-fatality are needed from different regions in the developing world. Estimates are likely to differ according to whether the region is experiencing epidemic *S. dysenteriae*, and so a diversity of settings must be studied. Moreover, because of the repeated observation that a major fraction of these deaths occur outside of a treatment setting, community-based studies are needed to define out-of-hospital mortality.

2. Study objectives

1) To estimate the indicence of treated *Shigella* diarrhoea episodes in the general population and in the following age groups: 0-59.9 months, ≥60 months.

2) To estimate the proportion of treated diarrhoea episodes associated with *Shigella* in the general population, and in the following age groups: 0-59.9 months, ≥60 months.

3) To estimate the distribution of *Shigella* diarrhoea episodes associated with each *Shigella* species and serotype.

4) To estimate the proportion of *Shigella* isolates that are resistant to antibiotics conventionally used for treatment of shigellosis.

5) To estimate the incidence of dysentery—associated mortality in the general population and in the following age groups: 0-59.9 months, ≥60 months.

3. Methods

3.1 Overview

These burden-of-illness assessments will employ one year of surveillance to estimate the burden of *Shigella* diarrhoea. Because of difficulties in estimating the burden of illness due to shigellosis, cited earlier, the studies will employ both treatment-centre-based and community-based surveillance techniques.

Because dysentery-related mortality is much rarer than *Shigella* diarrhoea, the population needed to estimate rates of dysentery-mortality will be much larger than that needed to estimate rates of *Shigella* diarrhoea. Accordingly, it is recommended that a relatively large "source" population be demarcated for estimation of dysentery-mortality, and that *Shigella*-morbidity be assessed for a smaller "subpopulation" within the source population.

It is recommended that a census of the source population be done just prior to the start of surveillance for *Shigella* diarrhoea. In addition to enumerating and characterizing the demographic characteristics of the target population the census should inquire about patterns of health care utilization for treatment of diarrhoea and about deaths in the household occurring during the past year. For the surveillance phase of the study all sources of medical care for diarrhoea in the targeted subpopulation should be placed under clinical surveillance, and all patients from the subpopulation who present for care at these settings should receive clinical and microbiological evaluations. At the end of the surveillance period, the census of the source population should be repeated. This census will enumerate deaths that occurred in the target population during the surveillance phase and will determine whether the deaths were preceded by diarrhoea or dysentery. The census will also inquire about recent diarrhoeal episodes that occurred in the family and will note the sequence of medical care sought for these episodes.

3.2 Background data needed in preparing proposals

For submission of a proposal, the following information should be considered: 1) the geographical boundaries (including a map) and sizes of the source and subpopulations; 2) the age (0-11.9 months, 12-59.9 months, 5-14.9 years, 15-40 years, >40 years) and gender distribution of these populations; 3) the stability of the populations (migration rates); and 4) the annual numbers and rates of deaths by age (0-11.9 months, 12-59.9 months, 5-14.9 years, 15-40 years, >40 years) in the source population. In addition, the sources of outpatient and inpatient care for diarrhoea in the subpopulation, both private and public, should be outlined.

Information should be provided about how accessible this care is to the population (geographically and financially), as well as about the extent to which the target population receives alternative care for diarrhoea (e.g., from pharmacists, traditional healers). Finally, a one-year retrospective review of records from these sources of care should be considered. This review should provide the number of diarrhoeal visits by age (grouped as for the census, above), characterized by type of diarrhoea (dysentery vs. non-dysentery) to enable calculation of rates of treated episodes of diarrhoea in the target population.

3.3 Preparation of the community

Efforts should be made before the project to obtain community participation in the project. Discussions about the study should be undertaken with community leaders in order to obtain their endorsement for the study, including encouragement of all residents with diarrhoea to seek first-line clinical care at one of the treatment sites being used for project surveillance. If active outreach to the community by community health workers is already in place, these workers should be encouraged to explain and endorse the project to their target populations. The census (*vide infra*) will provide another opportunity for explanation of the project to the target population and encouragement to seek firstline care of diarrhoea at a study treatment site.

3.4 Baseline census of the source population

At the outset of the study, a census of the source population should be conducted, giving, separately for each household, a listing of address of the household and the name of the head of household, and, for each resident, the person's name and age (in years for persons $\geq$24 months of age, or in months for younger persons) and gender. In addition, the names of all household members who died during the previous 12 months should be listed, together with person's age at death (in years for persons $\geq$24 months of age, or in months for younger persons), gender, date of death, and indication of whether the person had diarrhoeal symptoms in the illness leading to death, and, if so, whether the diarrhoea had visible blood. Information about these deaths should be obtained from a person who was with the decedent during the terminal illness, and, in the case of children, should be obtained from the child's caretaker. Finally, for persons residing in the target subpopulation, it should be noted for each individual whether diarrhoea occurred during the past month. If so, it should be noted whether the diarrhoea had stopped, how long the diarrhoea lasted, whether the character of the diarrhoea was (bloody vs. only non-bloody vs. non-bloody followed by bloody diarrhoea), and the names of all treatment centres where care was sought. This last information will be used to estimate the extent to which reliance upon *Shigella* episodes detected in treatment settings underestimates the burden of shigellosis in the community.

In order to keep track of subjects who present for care of diarrhoea, it will be desirable to assign each person in the census a unique identification number. One approach to assigning such numbers is to assign a consecutive number for each of the following: neighborhood of residence, household within the neighborhood, and person within the household. It is also useful to distribute to each household a household identification card, giving the number of the neighborhood and household, the household address; the name of the household head; and the names, ages, genders, and identification number of each resident. Residents should be encouraged to bring this card to a treatment site when presenting for medical care.

3.5 Treatment centre surveillance for diarrhoea

All treatment sites providing care for diarrhoea to the subpopulation should be equipped and trained to conduct surveillance for diarrhoea, to characterize diarrhoeal patients, and to obtain suitable faecal specimens for the diagnosis of *Shigella*. Surveillance in all sites serving the subpopulation shall be conducted for one year, beginning at the conclusion of the baseline census.

3.5.1 Clinical procedures

Every patient who presents to the treatment site for care of diarrhoea should have several pieces of information entered into a clinical log book. This book will be constructed as a line list, with each line corresponding to a single patient and with columns for entry of relevant information.

The log book might contain the following columns: running serial number in the book; (1) patient name; (2) patient age; (3) patient sex; (4) patient address; (5) name of patient's head of household; (6) patient's census identification number (if known); time and date of presentation for care; (7) pre-presentation duration of diarrhoea; (8) the maximum number of loose or liquid motions in any 24-hour period since the diarrhoea began; (9) the number of loose or liquid motions in the 24 hours before presentation; (10) whether the diarrhoea was ever watery; (11) whether the diarrhoea was ever bloody; (12) history of convulsion; (13) sensorium on presentation (e.g., coma, stupor, alert); (14) level of dehydration on examination (using criteria provided in *The treatment of diarrhoea: a manual for physicians and other senior health workers* (WHO/CDR/95.3)[1]); (15) evidence of rectal prolapse on examination; (16) evidence of gross marasmus on presentation; (17) evidence of pretibial edema on presentation; (18) specimen number of the rectal swab taken; (19) time and date of discharge; (20) status at discharge (alive or dead).

For patients who do not reside in the subpopulation, it would be sufficient to fill out the data only through entry 11; filling out this minimum amount of data on non-targeted patients may help ensure that data is filled out for every targeted patient.

Because of the large number of entries in the clinical log book, it will be necessary to use a bound book with large dimensions and to devote two consecutive pages to the line for each patient. Moreover, to facilitate computerized data entry, the information entered should be numerically coded wherever possible (e.g., 0 = no, 1 = yes, 9 = unknown for yes-no questions).

[1] Produced by the WHO Division of Diarrhoeal and Acute Respiratory Disease Control in October 1995. This manual - currently in its third revision - revises and replaces earlier versions WHO/CDD/SER/80.2 (1980) and WHO/CDD/SER/80.2 Rev 2 (1990). It is available on the Internet at http:\\www.who.int/chd/publications/cdd/textrev4.htm. To obtain a printed copy of the most recent version, please contact The Director, Department of Child and Adolescent Health and Development, Health Systems and Community Health, World Health Organizatin, Geneva, Switzerland.

To permit continuous computerized data entry of the recorded data, it is recommended that each treatment centre use two clinical log books. Book #1 would be used for patients who present for care during even numbered months and Book #2 would be used for patients who present for care during odd numbered months. When information is complete for patients entered in a given month, the book can be transferred for computerized data entry.

3.5.2 Treatment of patients

It is essential that the programme of surveillance be accompanied by training of health care personnel at the surveillance sites regarding appropriate management of patients with diarrhoea and dysentery.The *treatment of diarrhoea: a manual for physicians aand other senior health workers*[2] provides current WHO guidelines for appropriate management.

3.5.3 Collection and shipment of faecal specimens

It is crucial to laboratory investigations that care be taken to collect suitable faecal specimens, to use an appropriate transport medium, and to ensure that specimens are transported to laboratory in a timely manner [47-48]. Delays of more than one hour will often occur between stool collection and processing of stools in the microbiology laboratory. Because such delays can result in substantial underdetection of *Shigella* it is recommended that stool be collected either by rectal swab or by swabbing of freshly produced stool. Rectal or stool swabs, of which both sterile cotton or polyester-tipped swabs are acceptable, should be placed either in Cary-Blair semi-solid transport medium (C-B) or placed into buffered glycerol saline (BGS) transport medium. These media should be supplied to the treatment sites in screw-top tubes, and should be checked regularly in the treatment sites for evidence of desiccation (if desiccated, they should not be used).

To collect a rectal swab, a cotton-tipped swab should be moistened with transport medium (C-B or BGS). The swab should be inserted through the rectal sphincter, 1-2" into the anal canal, and rotated several times with a gentle “scrubbing” motion on the intestinal epithelium. The swab should be inspected for evidence of faecal material and placed into a tube of transport medium, ensuring that the head of the swab is submerged beneath the level of the medium. If a wooden applicator is used, the end portion of the swab stick that has been touched by the fingers should be broken off. The screw cap should be replaced tightly, and the inoculated medium should be stored in a refrigerator (4^{o}C).

Similar procedures for inoculation and storage of transport medium should be followed for swabs of freshly produced stools (stools that are not freshly produced are not suitable for this purpose). If mucus, blood or shreds of intestinal epithelium are present, these should be sampled with the swab. If stool swabs are used, care must be taken to use clean containers for collection of these stools in order to prevent cross-contamination between patients.

Each specimen should be labelled. It is suggested that the labels give the date of collection, the treatment site, the patient’s serial number in the clinical log book, and the patient’s initials.

[2] See footnote 1, page 11.

Specimens in transport medium should be shipped to the laboratory in insulated boxes with refrigerant packs designed to ensure refrigeration at (4°C), but avoiding temperatures that will freeze the specimens. If wet ice is used instead of refrigerant packs, care must be taken that water from the melting ice will not seep into the specimen tubes, or leak from the secondary container. If wet ice is used, it is recommended that the specimen containers be placed in waterproof plastic bags that can be tightly sealed. Drying of the specimen or not using a buffered transport medium will reduce the numbers of viable *Shigellae*.

Shipment of specimens to the microbiology laboratory should take place daily. Each shipment should be accompanied by a shipment log form. Prior to shipment the following information should be entered about the shipment: name of treatment site, time and date of shipment, total number of specimens in shipment; initials or name of person making the shipment. In addition, prior to shipment the following information should be entered onto the shipment log form about each specimen: name of patient, serial number of the patient in the clinical log book, date of collection of the faecal specimen. The form should also provide spaces for the following information to be filled out upon receipt of the shipment in the laboratory: date and time of receipt of the shipment; condition of the shipment (cold or warm; moist or dry); a space for indicating whether each listed specimen was in fact received; and the initials or name of the person receiving the shipment in the laboratory.

3.5.4 Supervision of surveillance

It is recommended that a trained physician-supervisor visit each treatment setting on a regular basis. At the beginning of the surveillance period, such visits should be frequent, e.g. once per week. Later, as the study progresses, judgement can be used in deciding to decrease the frequency of such visits, but in no case should it be less frequent than once every other week.

During the visits, the supervisor should check to make sure that the facility is equipped with all supplies necessary to conduct the surveillance. In this regard, it is particularly important to note the condition of transport medium and the method used for storage of faecal specimens prior to transmission of the specimen to the laboratory. The supervisor should inspect the clinical log to ensure that it has been completed properly for patients encountered since the last supervisory visit, and should also make note of any problems encountered. Of particular importance is notation of the proportion of patients from the target population for whom adequate, detailed identification was obtained (notation of the census number is best) and the proportion from whom a faecal specimen was obtained. If there are diarrhoeal patients present in the facility, the supervisor should reinterview the patients to verify the accuracy of the information noted in the clinical log book. Ideally, a structured supervisory form should be used to record problems noted during the supervisory visit. Finally, it is very important the supervisor compile a list of all faecal specimens obtained and their dates of collection (this should be the same as the dates of patient presentation); this list should be taken to the laboratory for recording of date of receipt of the specimens in the laboratory. Delays in transmission of specimens should be detected and corrected immediately, since they can result in diminished isolation of *Shigella* from the faecal specimens.

All supervisory forms should be checked on a regular basis by the principal investigator on a regular (e.g., weekly) basis so that corrective actions can be instituted as quickly as possible.

Regular meetings of personnel involved in the study should be instituted, so that any problems that may arise can be discussed and solutions identified.

3.5.5 Start-up of surveillance

It is likely that a two—to-three month period will be required for start-up before the one year of surveillance begins. This period should be devoted to training health workers and supervisors on the appropriate collection of data and of specimens, as well as on the appropriate care of patients with diarrhoea. It should also be devoted to conducting a "dry-run" of surveillance in the treatment sites in order to correct major problems before definitive surveillance begins.

3.6 Laboratory isolation of *Shigella*

It is envisioned that field sites conducting these burden of illness evaluations will be capable of isolating *Shigella* from submitted faecal specimens and of determining the species (serogroup) and antimicrobial susceptibility of each isolate [49-54]. *Shigella* isolates thus identified will be stored and shipped to a central reference laboratory for confirmation of species and for determination of serotype.

In order to ensure quality control for laboratory procedures, it is recommended that only one laboratory be used for *Shigella* diagnosis for specimens collected in the surveillance.

The phases of laboratory processing of the faecal specimens consist of the following: specimen accession to the laboratory; primary isolation and serogrouping of *Shigella* species; antimicrobial susceptibility testing of *Shigella* isolates; recording of laboratory results; and storage and shipment of isolates for further testing at the reference laboratory.

As indicated in 3.5.3, upon receipt of each shipment of specimens, the shipping log form for the shipment should be completed by the laboratory worker accessioning the shipment. The following information should be filled out upon receipt of the shipment in the laboratory: date and time of receipt of the shipment; condition of the shipment (cold or warm; moist or dry); a space for indicating whether each listed specimen was in fact received; and the initials or name of the person receiving the shipment in the laboratory.

Primary isolation procedures for *Shigella* are outlined in Appendix A, while procedures for determining antimicrobial susceptibility are described in Appendix B. It is recommended that *Shigella* isolates be tested for their susceptibility to a minimum of the following: ampicillin, trimethoprim/sulfamethoxazole, nalidixic acid, and ciprofloxacin.

All *Shigella* isolates should be stored and shipped periodically to a designated reference laboratory for determination of serotype. Procedures for storage and shipment of isolates are given in Appendix C.

It is recommended that a log book be kept for entry of results of all tests done on the submitted faecal specimens. This log book should take the form of a line list (like the clinical log book) and, in addition to laboratory results, should provide all the identifying information for the patient provided on the shipment log form, including the name of the treatment centre; the time and date of shipment; the name of the patient; the serial number of the patient in the clinical log book; and the date of collection of the faecal specimen. The dates for completion should be noted in conjunction with the test results. If *Shigellae* are isolated, the location(s) (e.g., box number and matrix location within the box) of stored isolates should be provided.

In order to facilitate continuous computerized data entry of laboratory results, it is recommended that two books be used for each laboratory. Book #1 would be used for specimens received during even-numbered months, and Book #2 would be used for patients who present for care during odd-numbered months. When information is complete for specimens entered in a given month, the book can be transferred for computerized data entry.

Quality control is exceedingly important for laboratory work. It is recommended that all *Shigella* isolates be sent to a central reference laboratory for confirmation. In addition, it is useful to maintain a panel of reference isolates, consisting of *Shigella* isolates of each species as well as several additional non-*Shigella* members of the family *Enterobacteriaceae*. At periodic (e.g., once every two weeks) intervals, the laboratory supervisor should submit a random pick of one of these reference isolates to laboratory technicians for evaluation. The technicians should be unaware of the identity of the reference isolates that are submitted for identification. Results of these evaluations should be entered into a quality control monitoring book, and problems identified should serve as a basis for further training or other necessary adjustments. Visits should be made regularly to the laboratory by a study supervisor to inspect these results, and also to review the completeness and consistency of data entered into the laboratory log books.

3.7 Close-out census

At the end of the one-year surveillance period, a repeat census of the source population should be conducted. To conduct this census, it is recommended that the census conducted before the study be computerized and printed into books, with one page devoted to each household. Each household page would give the name, age, sex, and identification numbers of each member. Additional data fields should be included for entry of the following information about each household member: whether the person had migrated out (and when); whether person had died (giving the date, whether the death was preceded by diarrhoea, and, if so, whether the diarrhoea was ever bloody); and, for persons in the target subpopulation, whether diarrhoea had occurred in the past month, whether the diarrhoea had stopped, how long the diarrhoea lasted, whether the character of the diarrhoea was (bloody vs. only non-bloody vs. non-bloody followed by bloody diarrhoea), and the names of all treatment centres where care was sought.

Spaces should also be included for addition of new household members and dates of in-migration. Moreover, blank pages should be included for addition of new households.

3.8 Data management

Data management consists of several phases. As noted in earlier sections, supervisory visits should be made on a regular basis to the treatment centres and to the laboratory. During these visits data entered into the log books should be reviewed for completeness and consistency, and problems noted should be recorded in supervisory books and discussed with study personnel.

On a regular basis, data should be transferred to the study office for computerized data entry. Data thus entered should include: census data; clinical log book data; laboratory log book data; and shipment log form data. It should be noted that there must be variables in each of these data files that enable linkage of one to another. For example, records from the clinical log books might be linked to the census with use of the census identification number, or, *in lieu* of such a number, the patient name, gender, age, address, and name of household head. Data entry programmes will be required for entry of data from each form and for automatic checking to determine that the entered data for each record are complete and consistent. Problems noted (missing data, inconsistent data) should be printed out; one or more members of a "data team" will then be responsible for updating those errors that can be resolved by inspecting the original data forms or by inspecting information from other data files. It also will be important to be able to link records from related data files. For example, for each treatment visit, it will be important to link the related census record, if it exists, as well as the related laboratory results record. Errors in linkage (missing linkage or linkage of records of different individuals) require detection and, if possible, resolution via correction of erroneous information in individual records.

All modifications of records done as part of these data-editing activities require documentation. It may be possible to employ a special "auditing" programme to record all changes. Failing this, all changes should be entered into a log book.

These data editing activities constitute a continuous process that requires systematic organization. A mechanism is required for batching sequential groups of records from each file and for declaring such batches as "clean" with respect to the completeness and consistency of individual records and to the linkage of records to records of related files. By the end of the study, an archivable set of data files that are declared suitably clean should result; data analysis should use this archivable file.

3.9 Data analysis

The analysis of disease burden will focus on the incidence of *Shigella* diarrhoea and on the incidence of dysentery-related deaths.

The following case definition can be used for *Shigella* diarrhoea. A diarrhoeal visit will be one in which $\geq$3 loose or liquid stools, or $\geq$1 loose or liquid motion with visible blood, were noted prior to presentation for care. Since more than one such visit could be made for a given episode of diarrhoea, it is useful to define a diarrhoeal episode as all sequential visits for which the date of discharge is $\leq$7 days before the onset of symptoms for the next visit. *Shigella*-diarrhoea is defined as a diarrhoeal episode in which *Shigella* is isolated in any component visit for the episode.

A dysenteric death can be defined as a death in which bloody diarrhoea occurred as a symptom for the illness leading to death.

The incidence of *Shigella* diarrhoea is calculated, for the target subpopulation, as the number of *Shigella* diarrhoeal episodes detected during the year of surveillance divided by the person-time at risk (approximated, in person-years, by the average of the number of persons in the baseline and close-out censuses).

The incidence of dysenteric death is calculated, for the target source population, as the number of dysenteric deaths noted in the close-out census among persons in the baseline census, divided by the by the person-time of follow-up contributed by the population in the baseline census. Person-time for this calculation is one year for each person present in both censuses, one half year for persons lost to follow-up between the censuses, and the time from onset of surveillance to death for persons who died during the surveillance period.

The proportion of such deaths that might be related to *Shigella* can be judged by the proportion of bloody diarrhoeal episodes detected in the treatment centres that are associated with *Shigella*.

Incidence rates within age-strata are calculated in an analogous fashion, taking the average number of persons within each stratum in the baseline and close-out censuses.

Data from the censuses can be used to augment these basic analyses.

For example, the extent to which non-use of medical facilities for treatment of diarrhoea augments the community-based incidence of *Shigella* diarrhoea can be estimated from the percentage of bloody vs. non-bloody diarrhoeas seeking care, by duration of the episodes and by age, and by augmenting *Shigella* episodes of different types (bloody vs. non-bloody), of different durations, and in different age groups accordingly. Moreover, the year-to-year variation in dysenteric mortality can be estimated by calculating separate incidence rates for deaths recalled retrospectively in the baseline census and for deaths recalled retrospectively in the follow-up census.

Additional analyses of interest would include the distribution of *Shigella* isolates by species and serotype, as well as the proportion of isolates of different species that are susceptible, of intermediate resistance, or fully resistant to antibiotics of interest.

3.10 Size of the required source population and subpopulation

The size of the population needed will be determined by the need to estimate the following with suitable precision: 1) the incidence of dysenteric mortality in persons <5 years and ≥5 years of age; and 2) the incidence of treated *Shigella* diarrhoea in persons <5 years and ≥5 years of age.

Calculation of these rates is described in Section 3.9. Note that estimation of person-years at risk differs for the incidence of diarrhoea and the incidence of death.

To estimate the number of person-years at risk (L) required during surveillance, it is necessary to estimate the anticipated incidence (per person-year) of the event (R) in the population and age-group of interest, and it is necessary to postulate the width

of the 95% confidence interval surrounding this incidence rate. This width is calculated as R $\pm$ 1.96 (S), where S is the standard error of the incidence.

If the age interval under consideration comprises N years, the number of person-years of observation needed (L) is given by:

$$L = \frac{R\ (2 - N*R)}{S^2\ (2 + N*R)}$$

It is then necessary to estimate the size of the population necessary to yield the calculated L, taking account of migration and mortality rates.

For dysenteric mortality, if the estimated incidence is 1 death/1000 person-years of follow-up of children <5 years of age, and the desired 95% confidence interval for this rate is $\pm$ 0.8 death/1000 person-years of follow-up, 6250 person years of follow-up will be required for this age group. If 6800 children are required for this follow-up and if children in this age range represent 13% of the population, the needed total population (all age groups) will be about 50 000 persons. If the estimated incidence is 1 death/10 000 person-years of follow-up of persons 5-80 years and the desired 95% confidence interval for this rate is $\pm$ 0.8 death/10 000 person-years of follow-up, 62 500 person years of follow-up will be required for this age group. If 68 000 persons are required for this follow-up and if persons in this age range represent 87% of the population, the needed total population (all age groups) will be about 78 000 persons. Thus, if rates are to be estimated for both age groups in the same source population, the source population assessed for mortality must have at least 78 000 persons.

The incidence of treated *Shigella* diarrhoea is likely to be substantially higher than the incidence of dysenteric mortality. A reasonable estimate for children <5 years would be 6 episodes per 1 000 person-years in this age group. If the desired 95% confidence interval for this rate is $\pm$ 4.8 episodes/1000 person-years of follow-up, 1000 person years of follow-up will be required for this age group. If the population is stable, 1000 children will be required for this follow-up (*vide supra*), and if children in this age range represent 13% of the population, the needed total population (all age groups) will be about 8000 persons. A reasonable estimate for the incidence in persons 5-80 years would be 0.4 episodes per 1000 person-years. If the desired 95% confidence interval for this rate is $\pm$ 0.32 episode/1000 person-years of follow-up, 10 000 person-years of follow-up will be required for this age group. If the population is stable, 10 000 persons in this age group will be required for this follow-up (*vide supra*), and if persons in this age range represent 87% of the population, the needed total population (all age groups) will be about 12 000 persons. Thus, if rates are to be estimated for both age groups in the same subpopulation, the subpopulation under surveillance for treated diarrhoeal episodes at least 12 000 persons.

These major differences in the size of the population needed for estimation of the rate of dysenteric mortality and for estimation of the rate of *Shigella* diarrhoea justify conducting the censuses in a large source population and conducting surveillance for diarrhoea in a smaller subpopulation that constitutes a part of the source population.

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Appendix A: Primary isolation

Overview of the genus *Shigella*

The genus *Shigella* is composed of nonmotile bacteria that belong to the family *Enterobacteriaceae*. They are oxidase negative, and with the exception of *S. flexneri* 6 do not form gas from fermentable carbohydrates. *Shigellae* are biochemically much less active than their close relatives, members of the genus *Escherichia*. Cultures of *S. sonnei* ferment lactose upon extended incubation, but other *Shigellae* do not utilize this substrate in conventional medium. Lysine is not decarboxylated, and ornithine is decarboxylated only by *S. sonnei* and *S. boydii*.

Enrichment

Enrichment for enteric pathogens is designed to detect small numbers of an organism by allowing the growth of the desired organisms while inhibiting the growth of normal enteric flora. An enrichment medium specifically formulated for *Shigellae* has not been developed. However, if enrichment is desired Gram negative (GN) broth may be used. GN broth allows the growth of *Shigellae* while inhibiting the growth of most non-pathogens. If used, GN broth should be subcultured at 6 to 8 hours after inoculation as longer incubation times allow the overgrowth of normal faecal flora, such as *E. coli*. Selenite and tetrathionate broth enrichment medium are not satisfactory for *Shigella* enrichment. Each laboratory should carefully evaluate recovery rates of *Shigellae* before and after the use of enrichment broth to determine its value.

Isolation of *Shigellae* from plating media

Shigellae can be isolated from a number of commercially prepared plating media used with members of the *Enterobacteraceae*. MacConkey (MAC), XLD, Hektoen (HEK), and *Salmonella-Shigella* (SS) and Deoxycholate Citrate agar (DCA) are among the more common selective/differential agar media used for the recovery of *Shigellae* (refer to Table 1). For optimum isolation of *Shigella* the use of XLD or Hektoen in combination with another enteric differential medium (MAC, SS, DCA) has been recommended (Enterobacteraceae, Rhoden). If GN or another enrichment broth is used, it should be inoculated and incubated at 35° C for the appropriate length of time (6-8 hours for GN broth) and then plated to the primary isolation medium with streaking for isolated colonies. Isolation medium is incubated at 35° C for 18-24 hours and examined for suspicious colonies. MAC, HEK and XLD should be inoculated with a small inoculum. DCA and SS are more inhibitory media and should be inoculated with a larger inoculum. After 18-24 hours of incubation *Shigellae* will appear as colorless to slightly pink colonies on MAC, SS, and DCA

and as green or blue-green colonies on HEK. It is recommended that up to three suspect colonies from the primary isolation media be selected and inoculated into a nonselective medium and for screening biochemical tests.

Table 1: Selective and Differential Plating Media for *Shigella*

Plating Medium	Fermentable Carbohydrate	Indicator	Fermenter	Non-fermenter
MacConkey Agar	Lactose	Neutral Red	Red	Colorless
Deoxycholate Citrate Agar	Lactose	Neutral Red	Red	Colorless
XLD Agar	Xylose Lactose Sucrose	Phenol Red and H_2S Indicator	Yellow	Pink to Red
Hektoen Enteric Agar	Salacin Lactose	Brom Thymol Blue and H_2S Indicator	Yellow-Orange	Green or Blue-Green
Salmonella and *Shigella* (SS) Agar	Lactose	Neutral Red and H_2S Indicator	Red	Colorless

Presumptive identification of *Shigella* with biochemical tests

Generally, the use of a few well-chosen biochemical screening tests will be sufficient to presumptively identify *Shigellae*. Care should be taken to insure that caps on tubes for all biochemical tests are loosened before incubation. The reactions of *Shigellae* in selected biochemical tests are found in Table 2.

Notes on biochemical tests

All biochemical tests should be performed with pure, 24-hour old cultures. Care should be taken to follow the directions for each test for inoculation, incubation, and final reading. Positive and negative controls should be examined along with the test specimens. Results of all tests, including those of the quality control cultures, should be recorded in a laboratory log or electronic database.

Oxidase test

The oxidase test is conducted with fresh growth from any non-sugar-containing medium. Two to three drops of oxidase reagent are placed on a piece of filter paper in a Petri dish. The culture is smeared across the wet paper with a platinum (NOT nichrome) loop, a clean wooden applicator stick, or tooth pick. A positive reaction results in the bacterial growth becoming dark purple in color within 10 seconds. Positive and negative controls should be tested at the same time. *Shigellae*, as part of the family *Enterobacteriaceae*, are all oxidase negative.

Table 2. Characteristics of Typical *Shigella* Isolates in Selected Biochemical Tests*

Test	Reaction
Oxidase	-
KIA *	K/A, no gas, no H_2S
Motility	-
Lactose (acid production)	-*
Glucose (acid production)	+
Glucose (gas production)	-*
Urease	-
Lysine	-
Ornithine	-* (+ with *S. sonnei)*
Citrate (Simmon=s)	-
Methyl Red	+
Voges-Proskauer	-

* See biochemical notes in following section

Kligler's iron agar (KIA)

KIA is a carbohydrate-containing screening medium that is widely used in diagnostic microbiology. The reactions of *Shigellae* after 18-24 hours incubation at 35°C in KIA are K/A, no gas, no H_2S (where K indicates alkaline, or red, coloration of the slant and A indicates acid formation, as demonstrated by yellow coloration in the butt of the slant). KIA slants are inoculated by stabbing the butt and streaking the surface of the medium. Slants should be aerobically incubated (loose caps) at 35°C. Accurate test results are obtained only if slants are read 18 to 24 hours after inoculation.

Triple sugar iron agar (TSI)

Because of the presence of sucrose in TSI, a similar enteric screening test medium, the reactions of rare *Shigellae* (particularly *S. flexneri*) may be A/A, no gas, no H_2S. However, the majority of *Shigellae* do not ferment sucrose, and many *Shigella* isolates on KIA will give a similar reaction to that of the TSI (K/A, no gas, no H_2S).

Gas from glucose

Certain biotypes of *S. flexneri* are able to produce small amounts of gas from glucose.

Lactose

Late lactose fermentation (fermentation after 24-48 hours) is common for isolates of *S. sonnei*. Rare isolates of *S. dysenteriae* and *S. flexneri* may also ferment lactose after prolonged incubation.

Lysine decarboxylase

A lysine iron agar slant or lysine broth may be used to test for the production of lysine decarboxylase by presumptive *Shigella* isolates. The lysine iron agar slant should be inoculated by stabbing the butt and streaking the slant. Lysine broth should be inoculated with a light inoculum from a young culture from a nonselective agar medium. *Shigellae* are negative for lysine decarboxylase.

Ornithine decarboxylase

An ornithine decarboxylase glucose agar slant or ornithine broth may be used to test for the production of ornithine decarboxylase. Ornithine glucose agar should be inoculated by stabbing the butt and streaking the slant. Ornithine broth should be inoculated with a light inoculum from a young culture from a nonselective agar medium. Most isolates of *S. sonnei* are able to produce ornithine decarboxylase. Rare isolates of *S. boydii* are ornithine positive. Other *Shigellae* are negative in this test.

Serogrouping of *Shigella* isolates

Shigellae are composed of four species or serogroups that are referred to by name as well as with the letters A, B, C and D (Table 3). Serogroup A refers to *S. dysenteriae*; B denotes *S. flexneri*; C corresponds to *S. boydii*; and D corresponds to *S. sonnei*. Although it is possible to identify *Shigella* species with biochemical tests alone, the usual practice is to presumptively identify them to the genus level with biochemical tests and then to confirm their identity with polyvalent (serogroups A-D) antisera. Serological evaluation of *Shigellae* below the species level is usually only done at a reference laboratories. *Shigella sonnei* is the only member of the species with one serotype.

Suspected *Shigella* isolates should be tested by slide agglutination with polyvalent A-D *Shigella* antisera. Isolates that agglutinate with polyvalent antiserum may be reported as presumptive *Shigella* species. These isolates should then be serogrouped with monovalent O (serogrouping) antisera. Agglutination tests for *Shigella* somatic "O" antigens are carried out in a petri dish or on a clean glass slide. Using a wax marking pencil, columns are drawn on the petri dish or glass slide in which suspensions of the test organism can be made in saline and antisera. A straight wire inoculating needle or sterile stick or tooth pick is used to remove a portion of the growth from the surface of a KIA or TSI slant or other non-inhibitory solid media. The growth is emulsified in a drop of physiological saline and mixed thoroughly by tilting back and forth for about 30 seconds. The suspension is examined carefully to ensure that it is even and does not show clumping due to autoagglutination. If clumping occurs, the culture is termed "rough," signifying that it cannot be serotyped. If the suspension is smooth (turbid and free-flowing), one loopful (or one drop) of antiserum is added, mixed well using the loop, and observed for agglutination against a dark background. If the reaction is positive, very strong clumping will appear within 30 seconds to 1 minute. If there is no agglutination, a heavy suspension of organisms in 0.5 ml of 0.85% NaCl is made, and heated for 15 minutes in a boiling water bath. the suspension is then cooled and retested with *Shigella* antisera. *Shigella* B and D will usually agglutinate live, whereas groups A and C as well as the Alkalescens-dispar group of *Escherichia coli* (an anaerogenic biotype that is confused with *Shigellae*) frequently must be heated.

Table 3: Serogroups of *Shigella*

Species	Serogroup	Serotypes
S. dysenteriae	A	1-10
S. flexneri	B	1-6 with subtypes, X,Y
S. boydii	C	15
S. sonnei	D	1

Appendix B: Antimicrobial susceptibility testing

Procedure for agar disc diffusion

Mueller Hinton agar medium should be prepared and autoclaved according to the manufacturer's directions. After the agar has been cooled to approximately 50°C in a water bath, the medium should be poured in 15 x 150 mm Petri dishes to a depth of 4 mm or more (approximately 60-70 ml per plate). The plates should be dried in an incubator before use.

A 0.5 McFarland turbidity standard should be prepared by adding 0.5 ml of 1.175% (w/v) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% sulfuric acid. The turbidity standard should be in a test tube identical to the one to be used to grow the test organism in broth. The McFarland standard should be sealed with wax, or some other means to prevent evaporation, and stored for up to 6 months at room temperature (22-25°C) in the dark.

Each culture to be tested for antibiotic susceptibility should be streaked onto a non-inhibitory agar medium (blood agar, brain-heart infusion agar, or trypticase soy agar) so as to obtain isolated colonies. After incubation at 35-37°C overnight, well-isolated colonies of the same morphological type should be selected. Growth should be harvested with an inoculating needle or loop, and the growth should be transferred to a tube of sterile broth (Mueller-Hinton broth, heart infusion broth, or trypticase soy broth). A sufficient quantity of bacterial growth should be emulsified in the broth so that the turbidity approximates that of the 0.5 McFarland standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which are drawn sharp black lines. If necessary, turbidity can be reduced by adding sterile broth. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile nontoxic swab should be dipped into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, the swab should be rotated to remove excess liquid. The swab should be streaked over the entire surface of the medium 3 times, each time rotating the plate approximately 60° after each application to ensure an even distribution of the inoculum. Finally, the swab should be streaked all around the edge of the agar surface.

The working supply of antibiotic disks should be kept in the refrigerator. On removal from the refrigerator, the containers should be left unopened at room temperature for about 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation that occurs when warm air reaches the cold container. If a disk-dispensing apparatus is used, it should have a tight-fitting cover and be stored in the refrigerator. It should also be allowed to warm to room temperature before using.

The antibiotic-containing discs should be applied to the plates as soon as possible after inoculation, but within 15 minutes. After the disks are placed on the plate, the plate should be placed in an incubator at 35-37°C for 18-24 hours.

After overnight incubation, the diameter of the zones of *complete* inhibition (including the diameter of the disk) are measured and recorded in millimeters. The measurements can be made with a ruler on the under-surface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (80% inhibition) should be ignored and the margin of heavy growth is measured. The zones of growth inhibition should be compared to a current zone-size interpretative table and recorded as susceptible, intermediate, or resistant to each drug tested.

Susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other variables. In order to obtain reliable test results, it is important to include control organisms with each test and to follow the procedure precisely.

Table 4: Zone Size Interpretative Standards for Selected Antimicrobial Disks

Antimicrobial Agent	Disc Potency (ug)	Zone Diameter (mm)		
		Resistant	Intermediate	Sensitive
Ampicillin	10	≤13	14-16	≥17
Ciprofloxacin	5	≤15	16-20	≥21
Norfloxacin[a]	10	≤12	13-16	≥17
Ofloxacin	5	≤12	13-15	≥16
Trimethoprim/ Sulfamethoxa-zole	1.25/ 23.75	≤10	11-15	≥16
Amoxicillin-Clavulanic acid	20/10	≤13	14-17	≥18

[a] Data for urinary tract infections only

From *Clin. Microbiol. Proc. Handbook*, ASM Press, 1995

Table 5: Zone Diameter Limits for Quality Control of Selected Antimicrobial Disks

Antimicrobial Agent	Disc Potency (ug)	Zone Diameter Limits (mm) for *E. coli* ATCC 25922
Ampicillin	10	16-22
Ciprofloxacin	5	30-40
Norfloxacin	10	28-35
Ofloxacin	5	29-33
Trimethoprim/ Sulfamethoxazole	1.25/23.75	24-32
Amoxicillin-Clavulanic acid	20/10	19-25

From Clin. Micro. Proc. Handbook, ASM Press, 1995

Appendix C: Storage and shipment of isolates

Storage of isolates

Pure growth of *Shigella* species will usually remain viable for several days on solid media held at room temperature (22-25°C) unless the media dries out or becomes acidic. However, if cultures are to be maintained for periods of time longer than a few days, they should be appropriately prepared for storage. Selection of a storage method will depend on the length of time the organisms are to be held, and the equipment and facilities available to the laboratory.

Short-term storage

Shigellae should never be stored on carbohydrate-containing medium, as acidic byproducts of metabolism will quickly reduce viability. Blood agar base (BAB), trypticase soy agar (TSA), heart infusion agar (HIA), nutrient agar (NA) and egg agar are all good storage media. The storage medium should be prepared and dispensed in 3 to 4-ml amounts in small tubes (approximately 13 x 100 mm) and then sterilized at 121°C for 15 minutes. While still hot, the tubes should be placed in a slanted position to provide a short slant and deep butt. The inoculating needle should be stabbed into the butt of the medium once or twice, and then the slant should be streaked. Incubation should be overnight at 35-37°C. The tube should be sealed with cork stoppers that have been soaked in hot paraffin or some other way that will provide a tight seal. Cultures should be stored at room temperature (about 22°C) in the dark. Sterile mineral oil may also be used to prevent drying of slants. Mineral oil (liquid petrolatum) should be sterilized in a hot air oven at 170°C for 1 hour. Sufficient sterile mineral should be added to cover the slants to 1 cm above the top of the agar. Subcultures should be made when needed by scraping growth from the slant. Strains maintained in pure culture in this manner will usually survive for several years.

Long-term storage

Frozen storage (-70°C) in sterile skim milk freezing medium:

a) Pure cultures of *Shigella* should be inoculated onto a TSA or HIA slant (or other growth medium) and incubated for 18-24 hours at 35-37°C.

b) Cells should be harvested from the slant and a suspension in sterile skim milk should be made.

c) The suspension should be dispensed into cryovials (freezing vials specially designed for use at very low temperatures).

d) The suspension should be frozen rapidly by placing the sealed vials in an alcohol-dry ice (frozen CO_2) bath until frozen. Transfer the frozen vials to a freezer at -70°C.

Recovery of cultures

a) Frozen cultures from the freezer should be placed into an alcohol-dry ice bath and transferred to laboratory safety cabinet, or clean area if cabinet is not available.

b) The top of the vial should be removed, being careful not to contaminate the top or inside of the vial.

c) Using a sterile loop, the top-most portion of the culture should be scraped and transferred to growth medium.

d) The vial should be reclosed before the contents completely thaw, and the vial should be returned to the freezer. With careful technique, transfers can be successfully made from the same vial several times.

Transport and shipment of cultures and specimens

Regulating organizations

The United Nations Committee of Experts on the Transport of Dangerous Goods develops recommended procedures for the safe transport of dangerous goods. The International Civil Aviation Organization (ICAO) has used these recommendations as the basis for developing the regulations for the safe transportation of dangerous goods by air. The regulations of the International Air Transport Association (IATA) contain all of the requirements of the ICAO Technical Instructions for the Safe Transport of Dangerous Goods. However, IATA has included additional requirements which are more restrictive than those of ICAO. Member airlines of the IATA have adopted the use of the IATA regulations governing dangerous goods, and shippers must comply with these regulations in addition to any applicable regulations of the State of origin, transit, or destination.

The shipment of infectious agents or diagnostic specimens by air must be in accordance with local, national and international regulations. International air transport regulations may be found in the ICAO publication entitled, "Dangerous Goods Regulations". This reference is published annually, in January. No attempt is made here to describe the existing regulations, as they may change from one year to the next. The reader is advised to obtain a copy of the IATA regulations from one of the regional offices given below. The IATA regulations are published in English, Spanish, French, and German.

Orders from North, Central and South America, Asia, Australia and the Pacific:

Publications Assistant	Telephone: (514) 844-6311
Intl. Air Transport Assn.	FAX: (514) 844-5286
2000 Peel Street	Telex: 05-267627
Montreal, Quebec	Cable: IATA MONTREAL
Canada H3A 2R4	Teletype: YULTPXB

Orders from Europe, Africa, and the Middle East:

Publications Assistant	Telephone: (22) 799.25.25
Intl. Air Transport Assn.	FAX: (22) 798.35.53
IATA Centre	Telex: 415586
Route de l'Aéroport 33	Cable: IATA GENEVA
P.O. Box 672	Teletype: GVATPXB
CH-1215 Geneva 15 Airport	
Switzerland	

Guidelines for packaging and labeling infectious substances

The following guidelines for packaging infectious substances are taken from the IATA publication "Dangerous Goods Regulations". They are presented as an example of acceptable packaging procedures for infectious materials. However, they may not reflect current national, state, or IATA requirements for packaging and labeling for infectious substances. For current information on packaging and labeling requirements, the reader is advised to consult the appropriate national and state regulations, and the current-year IATA publication entitled "Dangerous Goods Regulations".

Individuals who ship infectious agents or diagnostic specimens must comply with all local and international regulations pertaining to the packaging and handling of these items. They must ensure that specimens arrive at their destination in good condition and that they present no hazard to persons or animals during shipment.

Packaging guidelines for etiologic agents

The inner packaging must include:

1. An inner watertight primary container
2. A watertight, impact resistant secondary container
3. Absorbent material which must be placed between the primary container and the secondary container. If multiple primary containers are placed in a single secondary packaging, they must be wrapped individually to ensure that contact between them is prevented. The absorbing material, such as cotton wool, must be sufficient to absorb the entire contents of all primary containers.

The outer packaging must be of sufficient strength to protect and contain the contents. The outer container must be at least 100 mm (4 inches) in its smallest overall external dimension. An itemized list of contents must be enclosed between the secondary packaging and the outer packaging. Packages must be durably and legibly marked on the outside of the package with the address and telephone number of the consignee. A biohazard warning label must be affixed to the outside of the outer container, and must bear the inscription, "Infectious substance. In case of damage or leakage Immediately notify public health authority".