

Immunofluorescence Staining for Detection of Variola Virus

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In September 1975 Bangladesh was the only country in the world with endemic variola major, and the eradication of the disease was imminent. A rapid and accurate laboratory diagnostic method was required to supplement immunodiffusion in agar gel and culture on chorioallantoic membrane of embryonated egg available at the Institute of Public Health in Dacca, Bangladesh. To determine its effectiveness, a new, improved immunofluorescence (IF) staining technique was introduced. Laboratory specimens (scabs or vesicular or pustular impressions) were collected from patients who had, or were suspected of having, smallpox. Seventy-eight of 144 specimens collected were found to be IF positive for smallpox. As the number of laboratory-positive cases far exceeded the number of clinically diagnosed smallpox cases, IF-positive cases were reinvestigated and subsamples of the IF-positive specimens were tested at a World Health Organization poxvirus reference laboratory at the Centers for Disease Control in Atlanta, Ga. The results indicated 100% sensitivity for the IF technique (no false-negative results) in diagnosing variola major but also showed a high rate of false-positive results. Consequently, IF could not be recommended as a routine screening test for smallpox.

Bangladesh was the last smallpox-endemic country in Asia and the last country in the world with endemic variola major. In September 1975, there were only 31 known smallpox outbreaks, and the final eradication of the disease was in sight (11). To interrupt smallpox transmission, maximum containment was instituted in each suspect outbreak. To ensure optimum allocation of resources to those outbreaks with actual smallpox, rapid laboratory diagnosis was needed as a basis for discontinuing containment activities in those outbreaks that were not smallpox. The development by Kitamura et al. (6) of a rapid immunofluorescence (IF) staining technique adaptable to field conditions provided a potential breakthrough at a critical time in the smallpox eradication program in Bangladesh. This IF technique was designed for speed, accuracy, and adaptability to field conditions.

IF had been previously used in poxvirus diagnosis (4, 8) but had been discontinued because of false-positive results (2, 7, 9). The misdiagnosis of a chicken pox case as smallpox in Washington, D.C., in 1965 was by this method (1). This lack of specificity had reportedly been overcome through new methods of antiserum production; two cases of smallpox had been diagnosed in Japan by this refined technique (5),

and a preliminary field trial in Bihar, India, had been very encouraging (6). At the request of the World Health Organization (WHO), a team from the National Institute of Health in Tokyo, Japan, and the Tokyo Institute of Medical Sciences came to Bangladesh in September 1975 to further assess the practicability of the new technique.

MATERIALS AND METHODS

An IF microscope, antisera, and slides were flown to Dacca, Bangladesh, where an IF laboratory was established in the Poxvirus Diagnostic Laboratory at the Bangladesh Institute of Public Health. Local staff were trained to assist the WHO team. A standard form for clinical data was circulated to the field staff, with WHO specimen collection kits and written instructions. Clinical diagnoses were made by experienced paramedical staff who specialized in smallpox eradication activities, local medical officers, and WHO epidemiologists. Standard WHO clinical criteria for smallpox diagnosis were used (10). At that time culture on chorioallantoic membrane (CAM) and immunodiffusion in agar gel were the only smallpox laboratory diagnostic methods available in Dacca.

Smallpox program staff were directed to collect specimens from all suspect smallpox patients with vesicular or pustular eruptions. Ten scabs or two slides with vesicular or pustular impressions were collected from each patient and sent to the National Smallpox

Eradication Headquarters in Dacca. There, the specimens were coded and forwarded to the laboratory without clinical information. Immunodiffusion in agar gel and CAM, the standard methods heretofore available in Dacca, and IF were performed on all specimens in which material was sufficient for laboratory studies. The methods for IF antiserum preparation and use were those of Kitamura (5). The tests were carried out over a 1-month period.

RESULTS

Specimens were dispatched at ambient temperature without refrigeration. The average time of specimen transmission from the field to national headquarters was 2 days (range, 1 h to 5 days).

Of 205 specimens collected during the 1-month trial, 144 were adequate for laboratory diagnosis. Fifty-one specimens were excluded because of insufficient material, and 10 were excluded because of incomplete information. The results of the laboratory studies of the 144 specimens by clinical diagnosis are summarized in Table 1.

Eighteen of 18 clinically diagnosed smallpox cases (100%) were positive by IF. Sixty of 126 cases (48%) diagnosed as nonsmallpox rash disease were also positive for poxvirus by IF. By using standard agar gel and CAM techniques, a positive diagnosis for poxvirus was obtained in 7 of 18 (39%) of the clinically diagnosed smallpox patients and 2 of 126 (2%) cases of other rash disease.

As smallpox in Bangladesh was nearing eradication, it was imperative to resolve the apparent contradiction between clinical and laboratory diagnoses.

Reinvestigation of cases. In October 1975 a field program was organized to reinvestigate all 78 cases with IF-positive diagnoses. Simultaneously, diagnostic material available in sufficient quantity for further laboratory diagnoses was sent to the Atlanta, Ga., WHO laboratory without information on clinical diagnosis.

Of the 78 cases with IF-positive results, 68 were reexamined, with an average interval of 6

weeks between original specimen collection and reinvestigation. Ten cases could not be traced. Reinvestigation diagnoses, as determined by both clinical and epidemiological examinations, were compared with original diagnoses. Fifteen of the 18 cases diagnosed originally as smallpox were confirmed as smallpox on reinvestigation. Three cases of clinically diagnosed smallpox and 50 of 50 nonsmallpox rash cases were diagnosed as other rashes on reinvestigation. The diagnoses of "other rashes" included chicken pox (16 cases), scabies (3 cases), secondary syphilis (1 case), furunculosis (1 case). For 32 cases smallpox was ruled out, but no specific alternative diagnosis was established. No diagnoses of "other rash" were changed to "smallpox" upon reinvestigation.

To further assess the specificity of laboratory tests done in Dacca, as well as the reliability of clinical diagnoses, 27 specimens which were IF positive for poxvirus at Dacca were submitted for examination to the WHO Poxvirus Reference Laboratory at the Centers for Disease Control, Atlanta, Ga. Of the 27 specimens, 9, which were from the patients clinically diagnosed as having smallpox, were also positive for poxvirus by the reference laboratory. Of the nine positive specimens, five were positive by both electron microscopy and CAM, and four were positive by electron microscopy but negative by CAM. Eighteen specimens, which were from the patients clinically diagnosed as having other rashes but found to be IF positive for poxvirus in Dacca, were negative for poxvirus by electron microscopy and CAM at the reference laboratory.

DISCUSSION

Diagnostic techniques, clinical and laboratory, are assessed by their sensitivity and specificity. Using the 27 specimens submitted to the reference laboratory as a standard, we compared the sensitivity and specificity of original clinical diagnosis, field reinvestigation, IF, and Dacca laboratory (CAM and immunodiffusion in agar gel) findings (Table 2). Clinical diagnosis in Bangla-

TABLE 1. *Poxvirus laboratory results on 144 cases of vesicular or pustular Disease, Bangladesh, 1975*

Clinical diagnosis	No. of cases				
	Total examined	IF results		Dacca laboratory results	
		Positive	Negative	Positive for poxvirus ^a	Negative for poxvirus
Smallpox	18	18	0	7 (5 AG+ CAM+, 2 AG- CAM+)	11
Other rashes	126	60	66	2 (1 AG+ CAM-, 1 AG- CAM+)	124

^a AG+ or AG-, Positive or negative by immunodiffusion in agar gel; CAM+ or CAM-, positive or negative by CAM.

TABLE 2. Sensitivity and specificity of smallpox field diagnosis, field reinvestigation, IF, and Dacca laboratory immunodiffusion in agar gel and CAM, compared with diagnoses of the reference laboratory

Method	Sensitivity ^a	Specificity ^b
Initial field diagnosis	9 (100%)	18 (100%)
Field reinvestigation	9 (100%)	18 (100%)
IF	9 (100%)	0 (0%)
Dacca laboratory immunodiffusion in agar gel and CAM	4 (44%)	16 (89%)

^a Number of cases diagnosed as smallpox by the method in question out of nine cases diagnosed as smallpox by the reference laboratory.

^b Number of cases diagnosed as other rashes by the method in question out of 18 cases diagnosed as other rashes by the reference laboratory.

desh was both sensitive and specific in detecting smallpox (Table 2). This finding is further supported by the fact that 2 years of intensive posteradication surveillance, including examination of 400,000 Bangladeshis (0 to 19 years old) for smallpox facial scars, failed to uncover a single additional case of smallpox with onset after November 1975 (3).

Although IF was equally sensitive, the high rate of false-positives reduced the usefulness of this technique as a screening method for cases of suspected smallpox. The discrepancy in IF results between those reported here and those reported by Kitamura et al. (6) is difficult to explain. One possible explanation may be differences in the study population. In the earlier study in Bihar at least 55 of 78 cases (71%) were smallpox, whereas in Bangladesh only 13% were smallpox. A second possible explanation lies in the method of collection. In Bihar, specimens were collected and examined immediately or were frozen; in Bangladesh, specimens were transported at ambient temperatures. Noble and Loggins (9) have cautioned that adequate examination requires transport of specimens to the laboratory in frozen condition. The IF test, as used in this study, was not specific enough for routine field use in the diagnosis of smallpox. Electron microscopy and CAM remain the methods of choice for diagnosis of suspect smallpox cases. The results of this study apply to IF

identification of poxviruses by currently available methods. In other viral skin infections, IF has proven effective in differentiating viruses indistinguishable by electron microscopy, e.g., herpes simplex and varicella-zoster.

The inability of the Dacca laboratory to detect nonviable variola virus, identifiable only by electron microscopy, a technique not available in Dacca, is the most probable explanation for the low sensitivity of the Dacca laboratory procedures. This emphasizes the importance of including electron microscopy as a routine diagnostic tool in all suspect *Orthopoxvirus*-infected cases. The false-positive CAM case probably represents laboratory contamination, a constant hazard in field laboratories handling large numbers of specimens. The false-positive immunodiffusion in agar gel remains unexplained.

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