*Bull. Org. mond. Santé* }
*Bull. Wld Hlth Org.* } 1967, 37, 455-460

# A Transport Medium for Specimens Containing *Pasteurella pestis*

D. C. CAVANAUGH,[1] S. VIVONA,[1] DO-VAN-QÙY,[2] F. L. GIBSON,[3]
G. L. DEUBER[4] & J. H. RUST, JR[5]

*A medium, originally designed by Stuart and co-workers and later modified by Cary & Blair, for the maintenance and transport, without multiplication, of pathogenic bacteria contained in bacteriological specimens was tested in the laboratory and in the field in Viet-Nam to determine its effectiveness in preserving specimens known to contain* Pasteurella pestis.

*The results indicate that this medium should be useful in diagnostic plague studies in areas where transport facilities are inadequate. Properly collected clinical specimens, sent to a central laboratory by any means and under any climatic conditions likely to be encountered in the hot tropics, should yield viable* Pasteurella pestis *for at least 30 days.*

The problems associated with the bacteriological processing of specimens which arrive at a laboratory hours or days after collection were studied by Stuart et al. (1954). They were particularly interested in specimens containing gonococci and designed a special transport medium for this purpose. This medium, they pointed out, was useful for other pathogenic bacteria. These authors achieved three important objectives with their medium: (1) removal of an inhibitory substance or factor which is a constituent of the agar; (2) prevention of oxidation and desiccation of the specimen; and (3) prevention of unwanted multiplication of contaminating organisms.

Barlow et al. (1955) found Stuart's medium to be extremely useful for transporting specimens collected from the throat and urethra and reported the survival of several genera of pathogens. They also reported several modifications of Stuart's formula which could be made if not all the basic materials for Stuart's medium were not available.

Cary & Blair (1964) reviewed their experience and that of other workers with Stuart's medium for the transportation of faecal specimens. They noted that stool specimens transported in the medium frequently showed overgrowth of enteric pathogens by saprophytes. To obviate this problem, they introduced a modification of the basic Stuart formula. The Cary-Blair formula has been successfully used for the study of enteric disease by several groups of workers (Gaines et al., 1965; Cary et al., 1965; Vivona et al., 1966).

Since plague outbreaks often occur in areas far removed from adequate bacteriological laboratories, a transport medium which can maintain *Pasteurella pestis* in clinical specimens viable for long periods would be extremely useful.

There are, of course, many fully reliable methods for transporting viable *P. pestis* to the laboratory (Girard, 1952; Pollitzer, 1954; Goldenberg et al., 1964). But many of these methods depend upon sources of dry or wet ice, liquid nitrogen, etc, and upon the availability of regular sources of transport for unlimited weight. Where transport is irregular or sketchy, or where sources of supply are distant and unreliable, other possibly less satisfactory measures need to be employed. We have therefore studied both Stuart's and Cary-Blair's media for their effectiveness in preserving specimens known to contain *P. pestis* (*a*) by quantitative enumeration of viable plague organisms recovered by ordinary bacteriological methods from the media and (*b*) by determi-

[1] US Army Medical Research Team (Walter Reed Army Institute of Research), Viet-Nam. Present address: US Army Medical Component, SEATO (Bangkok), APO San Francisco 96346, USA.

[2] Institut Pasteur, Saigon, Viet-Nam.

[3] US Army Medical Research Team (Walter Reed Army Institute of Research), Viet-Nam, USA Drawer 100, APO San Francisco 96307, USA.

[4] US Army Medical Research Team (Walter Reed Army Institute of Research), Viet-Nam. Present address: MFSS Co. B, 1st Bn, Fort Sam Houston, Texas, USA.

[5] Department of Bacteriology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20012, USA.

nation of any change in the virulence of the organisms which might interfere with isolation by animal inoculation methods.

These two media were found to be equally useful in maintaining plague organisms viable under laboratory conditions. For studies with specimens from clinically suspect cases of plague under field conditions in Viet-Nam, the Cary-Blair formula was selected, partly because of its previously demonstrated usefulness in enteric work and partly because of the results of concomitant studies of diarrhoeal disease in Viet-Nam reported elsewhere (Vivona et al., 1966).

## MATERIALS AND METHODS

*Preparation of media.* Both media were prepared exactly as described by Stuart et al. (1954) and by Cary & Blair (1964).

Cary-Blair medium is prepared in chemically clean glassware rinsed with Sorenson's 0.067M buffer (pH 8.1). To 991 ml of demineralized distilled water the following are added in the order listed:

| | |
|---|---|
| Sodium thioglycollate | 1.5 g |
| $Na_2HPO_4$ | 1.1 g |
| NaCl | 5.0 g |
| Bacto agar (Difco) | 5.0 g |

The medium is carefully heated until it just becomes clear; it is then cooled to 50°C. 9 ml of freshly prepared aqueous 1% $CaCl_2$ are then added and the pH adjusted to 8.4. Excessive heating must be avoided. 7 ml of medium are dispensed into previously rinsed and sterilized 9-ml screw-capped vials. These are steamed for 15 minutes and allowed to cool, and the tops are then tightened.

Stuart's charcoal-tipped swabs, as used in Viet-Nam, are prepared by dipping cotton-tipped applicator sticks into a 1% suspension of charcoal in Sorenson's phosphate buffer (pH 8.1) that has been momentarily brought to boiling-point. The swabs are drained, placed in test-tubes or glassine envelopes and autoclaved. After sterilization, the swabs are allowed to dry at room temperature.

*Laboratory testing of transport media.* To show survival of *P. pestis* in transport media, *P. pestis* strains EV 76 or 195/P were inoculated into 100 ml of brain heart infusion broth (Difco) in a 500-ml Erlenmeyer flask and incubated for 48 hours at 37°C. To simulate clinical specimens, sterile charcoal swabs, previously cut to proper lengths, were added to the flask. The flask and contents were then shaken vigorously for 5 minutes on a rotary shaker. The swabs were then removed with sterile forceps and pushed into vials of the two semi-solid transport media. Caps were then replaced on the vials and the vials were stored at room temperature. A deliberate attempt was made to hold all specimens in transport medium in the hottest portion of the laboratory. Temperatures were always over 25°C and usually in excess of 33°C during the course of the day. For some experiments, suspensions of ground spleens obtained from moribund guinea-pigs which had been inoculated with virulent *P. pestis* were substituted for the broth cultures. Swabs were removed at various times from the transport media with sterile forceps, used to inoculate blood agar plates for direct bacteriological isolation, and then placed in 9 ml of saline contained in a 15-ml screw-capped tube. The tube with swab in saline was then agitated vigorously on a Vortex mixer and this tube was then considered to be a $10^{-1}$ dilution of the material contained on the swab. Further serial, decimal dilutions were made from this $10^{-1}$ dilution and aliquots of these were spread on the surfaces of blood agar plates for standard plate counts.

To test the virulence of *P. pestis* in the transport medium, 0.2 ml of the $10^{-2}$ dilution was inoculated subcutaneously into each of 4 white mice.[1]

For other experiments, specimens were taken from the heart blood of experimentally inoculated mice and placed in transport media. These were tested only for successful isolation, usually after being held at room temperature for one month.

*Field testing of Cary-Blair medium.* In the field, the multipurpose Cary-Blair medium was selected for actual use. This medium was used with Stuart's charcoal-tipped swabs since the charcoal was required to adsorb the inhibitory substances contained in agar.

The method recommended by Smadel et al. (1954) was employed for the collection of bubo material from suspected human cases of plague. The skin surface was rubbed with iodine; 0.2 ml of sterile physiological saline in a 2-ml syringe with a 23-gauge needle was injected into the bubo and the piston of the syringe gently manipulated to wash the saline and bubo contents in and out of the syringe several times. The syringe was then filled as much as possible and the needle withdrawn from the bubo. The contents of the syringe were then allowed to flow

[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

TABLE 1
SURVIVAL OF *PASTEURELLA PESTIS* STRAIN EV 76 IN STUART'S TRANSPORT MEDIUM [a]

| Experiment No. | Day tested | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 3 | 4 | 7 | 14 | 28 |
| 1 | $4 \times 10^6$ | $5 \times 10^6$ | $3 \times 10^6$ | $2.7 \times 10^6$ | $3.4 \times 10^6$ | — | — |
| 2 | $9 \times 10^5$ | — | — | — | $7.2 \times 10^5$ | $9.4 \times 10^5$ | $1.3 \times 10^5$ |
| 3 | $3 \times 10^6$ | — | — | — | $5.0 \times 10^6$ | $1.2 \times 10^6$ | $0.1 \times 10^6$ |

[a] Results expressed as viable plague bacilli per swab; — = not tested.

over the surface of the Cary-Blair transport medium. This material on the surface was then soaked upon a charcoal-tipped swab (Stuart et al., 1954). The swab was gently pushed into the medium, the protruding portion of the swab-stick broken off, and the screw-capped vial tightly sealed. The minute amount of material remaining in the syringe was used to make a smear, which was air-dried and immediately fixed in absolute methanol for 5 minutes. Extreme care was taken with these procedures to avoid the production of an aerosol.

At the laboratory, smears were stained with Wayson's stain and examined for plague bacilli. Swabs were removed from the transport medium with sterile forceps and used to inoculate tubes of brain heart infusion broth (Difco) and blood agar plates. Each swab was then placed in 1 ml of physiological saline and the fluid was agitated on a Vortex mixer. This saline was then taken up with a syringe and inoculated subcutaneously into 2 white mice. Mice were observed until time of death, survivors being sacrificed 14 days after inoculation. All mice were autopsied and examined by smear and culture. *P. pestis* was identified by the methods outlined by Baltazard et al. (1956).

The first opportunity to test the Cary-Blair medium under field conditions was encountered in Viet-Nam. It was customary to place lymph-node aspirations in approximately 5 ml of isotonic saline and to send this material to a central laboratory. Failures to isolate *P. pestis* from specimens of many patients who had clinical plague and from whom apparently adequate specimens were collected had been noted (Nguyen-Van-Ai, 1963). The Cary-Blair holding medium was therefore compared with the saline technique. Duplicate specimens of lymph-node aspirations from 40 Viet-Namese patients with adenitis were placed in the transport medium and in physiological saline. The specimens were then sent to the laboratory by the means routinely used by the clinicians concerned.

## RESULTS

Table 1 summarizes data from experiments when *P. pestis* was obtained from broth cultures and inoculated into Stuart's medium, and Table 2 the results of similar experiments with Cary-Blair medium.

TABLE 2
SURVIVAL OF *PASTEURELLA PESTIS* STRAIN EV 76 IN CARY-BLAIR TRANSPORT MEDIUM [a]

| Experiment No. | Day tested | | |
|---|---|---|---|
| | 0 | 7 | 42 |
| 1 | $7.0 \times 10^6$ | $7.0 \times 10^6$ | $2.0 \times 10^5$ |
| 2 | $5.5 \times 10^6$ | $6.5 \times 10^6$ | $3.5 \times 10^5$ |

[a] Results expressed as viable plague bacilli per swab.

These experiments show that *P. pestis* survives without multiplication in both transport media for at least 28 days.

When *P. pestis* was obtained from ground spleens of infected guinea-pigs and held in either transport medium for 30 days, the results were essentially similar to those obtained with broth cultures. The data from one such experiment are given in Table 3. Viable counts showed no appreciable loss of *P. pestis* 28 days after the specimens had been placed in transport media.

*P. pestis* was readily isolated from blood agar plates inoculated by swab prior to the counting pro-

TABLE 3
SURVIVAL OF *PASTEURELLA PESTIS* STRAIN 195/P COLLECTED FROM SUSPENSION OF GROUND SPLEEN FROM PLAGUE-INFECTED GUINEA-PIGS IN STUART'S AND CARY-BLAIR TRANSPORT MEDIA

| | Stuart's medium | | Cary-Blair medium | |
|---|---|---|---|---|
| | Day 0 | Day 28 | Day 0 | Day 28 |
| Viable bacilli per swab | $7.8 \times 10^5$ | $2.4 \times 10^5$ | $7.2 \times 10^5$ | $4.3 \times 10^5$ |
| Growth of *P. pestis* on blood plate inoculated with swab | Heavy | Heavy | Heavy | Heavy |
| Outcome of inoculation of mice with $10^{-2}$ dilution [a] | 4/4 | 4/4 | 4/4 | 4/4 |

[a] No. dead/No. inoculated

cedures; mice receiving 0.2 ml of the $10^{-2}$ dilutions all died of plague within 8 days.

In other experiments, specimens of heart blood collected on charcoal-tipped swabs from bacteraemic mice dying of plague were tested. *P. pestis* strains from India (195/P), Iran (PKR 108), Madagascar (111) and the USA (Miller) as well as 2 strains recently isolated from Viet-Nam were each injected into 2 white mice. All the infected mice died, and specimens were taken on charcoal-tipped swabs and placed in Cary-Blair transport medium. The specimens were held at room temperature for approximately one month (27-35 days); average room temperature was over 26°C throughout the period. The swabs were removed and treated as described above for material collected from human buboes. In all these cases, too, *P. pestis* was readily isolated from the blood plates inoculated with the swabs, all the plates showing profuse growth of plague bacilli. Again, all white mice receiving subcutaneous inoculations of the resuspended specimen rapidly died of plague.

Duplicate specimens from 40 Viet-Namese patients with adenitis were placed in saline and in Cary-Blair transport medium and sent to the laboratory. The results are given in Table 4. *P. pestis* was isolated from 23 of the specimens in transport medium but from only 16 of those in saline. In no instance was the saline positive and the transport medium negative. Most of the failures with saline were with specimens that had been in transit for several days between the place of collection and the laboratory.

The results of these tests indicated that an enhanced isolation rate could be expected if transport medium were substituted for the saline. Accordingly, kits of equipment were distributed to interested clinicians throughout Viet-Nam. Each kit contained a sterile, disposable syringe equipped with a 23-gauge needle, a vial of sterile saline, a vial of transport medium, a sterile charcoal-tipped swab and a microscope slide. Over 1000 such kits were distributed, and 141 specimens collected from Viet-Namese patients with adenitis were returned to the laboratory during the period covered by this report. *P. pestis* was isolated from 74 of those 141 specimens. In one noteworthy instance, *P. pestis* was isolated from 6 of 7 specimens which had been delayed in the mail for approximately 3 months.

A direct smear accompanied the specimen for culture in 124 instances. The results of examination of smears were compared with the results of culture. In several cases *P. pestis* was isolated from culture specimens whose corresponding direct smears yielded

TABLE 4
RELATIVE EFFICIENCY OF TRANSPORT MEDIUM AND SALINE IN MAINTAINING *PASTEURELLA PESTIS* (RESULTS WITH 40 DUPLICATE SPECIMENS)

| | | Transport medium | | Total |
|---|---|---|---|---|
| | | *P. pestis* isolated | *P. pestis* not isolated | |
| Saline | *P. pestis* isolated | 16 | 0 | 16 |
| | *P. pestis* not isolated | 7 | 17 | 24 |
| Total | | 23 | 17 | 40 |

TABLE 5

RESULTS OF DIRECT SMEAR AND CULTURAL EXAMINATION [a] OF SPECIMENS FROM PATIENTS WITH BUBOES

| | | Culture | | Total |
|---|---|---|---|---|
| | | *P. pestis* isolated | *P. pestis* not isolated | |
| Direct smear | *P. pestis* observed | 39 | 0 | 39 |
| | *P. pestis* not observed | 19 | 66 | 85 |
| Total | | 58 | 66 | 124 |

[a] Specimens received in Cary-Blair transport media.

no detectable bacilli. However, in no case where examination of the direct smear revealed *P. pestis* was there failure to isolate the organism from the specimen on transport medium (Table 5).

It should be emphasized that these specimens were collected by workers other than ourselves and there is no information as to the criteria for the selection of patients as bubonic plague suspects. Several controls were, however, incorporated into the system. The first was obtained through the examination of the direct smears of the lymph-node aspirates prepared by the physicians at the time of collection. And a further control was obtained by the inoculation of a white mouse with material from each specimen. In no instance was *P. pestis* isolated from white mice and not isolated by cultural means from the same specimen.

Owing to a shortage of animal facilities, $LD_{50}$ data on the Viet-Namese isolations were not attempted. Mice, however, were often observed to die of plague before cultural isolation of *P. pestis* was obtained and no diminution of virulence appeared to result from holding specimens in transport medium. *P. pestis* was not isolated by mouse inoculation from any culturally negative specimen.

## CONCLUSION

The Cary-Blair transport medium used with Stuart's charcoal-tipped swabs appears adequate to maintain virulent *P. pestis* in viable condition for at least 30 days under the conditions obtaining in the hot, wet tropics. Where sources of supply are scarce and inadequate and where transport is unreliable, the use of Cary-Blair transport medium for specimens from which *P. pestis* is to be isolated should result in an enhanced isolation rate.

## RÉSUMÉ

Les recherches, menées au Viet-Nam, décrites dans cet article mettent en relief les avantages de l'emploi des milieux de Stuart et de Cary-Blair pour le maintien de la vitalité de *Pasteurella pestis* lors de l'envoi au laboratoire d'échantillons de produits pathologiques.

Au laboratoire, *P. pestis* survit dans ces milieux pendant 28 jours au moins sans se multiplier et sans réduction appréciable du nombre de germes, malgré une exposition délibérée à la température ambiante variant de 25°C à plus de 33°C. Après ensemencement des mêmes milieux, dans des conditions proches de celles de la pratique, par diverses souches obtenues chez l'animal infecté, *P. pestis* est isolé sans difficulté après 30 jours et fait preuve d'une virulence intacte lors de l'inoculation à la souris. Aux fins de comparaison, des prélèvements pathologiques recueillis chez 40 sujets atteints d'adénite ont été expédiés au laboratoire à la fois sur milieu de Cary-Blair et en soluté salin. On a isolé *P. pestis* de 23 des échantillons dans le premier cas, de 16 spécimens seulement dans le second cas.

L'équipement nécessaire à l'emploi de cette méthode a été mis à la disposition d'un certain nombre de médecins viet-namiens: 141 envois de matériel pathologique ont été reçus jusqu'à présent au laboratoire, et *P. pestis* a pu être isolé de 74 d'entre eux, y compris 6 envois (sur 7) dont l'acheminement a duré environ trois mois.

## REFERENCES

Baltazard, M., Davis D. H. S., Devignat, R., Girard, G., Gohar, M. A., Kartman, L., Meyer, K. F., Parker, M. T., Pollitzer, R., Prince, F. M., Quan, S. F. & Wagle, P. (1956) *Bull. Wld Hlth Org.*, **14**, 457-509

Barlow, J. L., Yamakawa, Y. & Cavanaugh, D. C. (1955) *Jap. J. med. Sci Biol.*, **8**, 171-174

Cary, S. G. & Blair, E. B. (1964) *J. Bact.*, **88**, 96-98

Cary, S. G., Fusillo, M. H. & Harkins, C. (1965) *Amer. J. clin. Path.*, **43**, 294-296

Gaines, S., Haque, S. U., Paniom, W., Duangmani, C., Cary, S. G. & Blair, E. B. (1965) *Amer. J. trop. Med. Hyg.*, **14**, 136-140

Girard, G. (1952) *Bull. Wld Hlth Org.*, **5**, 109-116

Goldenberg, M. I., Quan, S. F. & Prince, F. M. (1964) *Bull. Wld Hlth Org.*, **30**, 741-746

Nguyen-Van-Ai (1963) *Inst. Pasteur Vietnam, Rapp.*, pp. 34-56

Pollitzer, R., (1954) *Plague*, Geneva (*World Health Organization: Monograph Series*, No. 22)

Smadel, J. E., Goodner, K. & Woodward, T. E. (1954) *The control of plague.* In: *Recent advances in medicine and surgery*, Washington, D.C., Army Medical Graduate School, Walter Reed Army Institute of Research, pp. 234-244

Stuart, R. D., Toshach, S. R. & Patsula, T. M. (1954) *Canad. J. publ. Hlth*, **45**, 73-83

Vivona, S., Minh-Han, T. T., Gibson, F. L. & Cavanaugh, D. C. (1966) *Milit. Med.*, **131**, 68-71