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Establishment of a Particle-Counting Method for Purified Elementary Bodies of Chlamydiae and Evaluation of Sensitivities of the IDEIA Chlamydia Kit and DNA Probe by Using the Purified Elementary Bodies

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To evaluate the sensitivity of commercially available test kits for detection of chlamydiae, we established a method of purifying *Chlamydia trachomatis* and *Chlamydia pneumoniae* elementary bodies (EBs). We then subjected the purified EBs, together with the purified EBs of *Chlamydia psittaci*, to the IDEIA Chlamydia (IDEIA) and DNA probe test kits to determine the EB numbers at the detection limits. The sensitivities of the test kits were thus compared. The results can be summarized as follows. (i) Intact EBs in the purified preparations were present at 100, 96.3, and 97% for the *C. psittaci* Cal 10, *C. trachomatis* L_2/434/Bu (L_2), and *C. pneumoniae* TW-183 strains, respectively. The preparations of the L_2 and TW-183 EBs contained a few EB envelopes, which reacted with antilipopolysaccharide monoclonal antibodies, as did the intact EBs, indicating that elimination of EB envelopes is not required for testing of the IDEIA kit's sensitivity. (ii) We established a method of counting intact EBs and EB envelopes under a scanning electron microscope after sedimentation of EBs on a coverslip by centrifugation. (iii) The EB numbers per assay at the cutoff level, which is set up in the IDEIA kit, were 9.6×10^2, 6.5×10^3, and 2.5×10^4 for the L_2, TW-183, and Cal 10 strains, respectively. When the same EB preparations were applied to the DNA probe kit, the EB number at the cutoff level was 7.5×10^3 per assay for the L_2 strain, but no reaction occurred for the Cal 10 and TW-183 strains at any EB number, indicating that the DNA probe kit is highly specific for *C. trachomatis*. Although the IDEIA kit designed for detection of *C. trachomatis* showed a sensitivity superior to that of the DNA probe, the chlamydial species was not determined by the IDEIA kit.

Recent investigations of chlamydial infections, especially the sexually transmitted diseases caused by *Chlamydia trachomatis* (19) and respiratory tract infections caused by *Chlamydia pneumoniae* (6), revealed the epidemiological status of chlamydial infections which should not be ignored (4, 8). Since the chlamydial organisms, including *Chlamydia psittaci* (19), are highly susceptible to macrolides and tetracyclines during their intracellular multiplication (10), the diseases caused by the organisms can be completely cured by medication with the proper antibiotics, provided that a reliable diagnosis is made. Therefore, the detection of the pathogens is important for medication purposes.

There are many commercially available kits for detecting chlamydiae, such as Cultureset (Ortho Diagnostic Systems Inc.), MicroTrack (Syva Co.) (26), Chlamydiazyme (Abbott Co.) (9), DNA probe (Gen Probe Co.) (13, 22, 30), and IDEIA Chlamydia (Dako Diagnostics Co.) (13, 23). The IDEIA Chlamydia (IDEIA) kit and DNA probe were established for the detection of the lipopolysaccharide (LPS) (23) and rRNA (22, 30) of *C. trachomatis*, respectively. Both test kits are now under evaluation in Japan.

Irrespective of the species differences, chlamydiae proliferate through a unique life cycle in which there are infectious elementary bodies (EBs) and noninfectious, reproductive reticulate bodies (RBs). Because of the low level of synchrony in the multiplication stage, EBs and RBs are released at the time when the host cells disintegrate during the late stage of infection. The infectious EB progeny alone proceed to perpetuate the infection by initiating a new cycle. Both EB and RB possess LPSs and ribosomes (2, 20, 28), and the LPSs and ribosomes contained in RBs are reactive in the IDEIA and DNA probe test kits, respectively (15, 16). RBs range in size from 0.5 to 1 μm, occasionally reaching more than 2 μm in diameter, and are regularly larger than EBs, which are uniform in size (approximately 0.3 μm in diameter). These facts suggest strongly that the contents of LPS and ribosome are constant in EBs, but not in RBs. This made us suspect that the sensitivities of both test kits, the IDEIA and DNA probe kits, should be able to indicate the number of EBs as a test parameter, but not to indicate the infectivity of a crude preparation containing infectious EBs and noninfectious RBs.

We established a method of purifying *C. trachomatis* and *C. pneumoniae* EBs, as well as a method for counting EBs in the purified preparation. By using the number of EBs as the test parameter, the sensitivities of the test kits were examined.

MATERIALS AND METHODS

Chlamydial strains. *C. trachomatis* L_2/434/Bu (L_2) (25), *C. psittaci* Cal 10 (5), and *C. pneumoniae* TW-183 (7) strains were used throughout the study. The L_2 strain was supplied by S. Yamasaki, National Institute for Health, Japan, and was maintained continuously in HeLa 229 or L-929 cells. The Cal 10 strain has been maintained in our laboratory for more than 20 years in monolayer or suspension cultures of L-929 cell lines. The TW-183 strain was purchased from Washington Research Foundation, Seattle, and was cultured

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in HeLa 229 cells by the method of Kuo et al. (11, 12). The Cal 10 and L_2 strains were cultured in suspended L cells by the method of Tamura and Higashi (27), and the strains were harvested on days 2 and 3 postinoculation, respectively. Cultivation of the TW-183 strain was carried out in HeLa 229 cells by the method of Kuo et al. (11, 12), and the strains were harvested on day 3 postinoculation.

Purification of EBs. The Cal 10 EBs were purified by the method of Tamura and Higashi (27), but trypsin treatment was omitted to exclude any possible change in the antigenic property of the EB surface. Our preliminary experiments indicated that EB preparations of the L_2 and TW-183 strains purified by the method of Tamura and Higashi (27) or Caldwell et al. (3) contained a number of RBs and their fragments. Therefore, we adopted the following new method for the purification of L_2 and TW-183 EBs. At 72 h postinoculation, infected cells were collected in sucrose-phosphate-glutamate (SPG) buffer and were homogenized with a Teflon homogenizer. After brief centrifugation at 900 × *g* for 10 min at room temperature to remove cell debris, the supernatant obtained was layered onto a two-layer cushion (bottom layer, 50% [wt/vol] sucrose solution; top layer, 30% [vol/vol] Urografin [3,5-diacetamido-2,4,6-triisobenzoic acid; Schering AG, Berlin/Bergkamen, Germany] in 30 mM Tris-HCl buffer [pH 7.3]); and the layered supernatant was then centrifuged at 8,000 × *g* for 60 min at 4°C with an RPS-25 swing rotor (Hitachi, Tokyo, Japan). The precipitate and the turbid bottom layer were suspended together in SPG buffer and were centrifuged at 12,000 × *g* for 30 min at 4°C. After resuspending the precipitate in SPG buffer and exposing it to DNase and RNase (final concentrations, 20 μg/ml each) for 30 min at 37°C, the suspension was layered onto a continuous Urografin gradient column (40 to 52% [vol/vol]) and was centrifuged at 8,000 × *g* for 60 min at 4°C. Two distinct bands were formed in the gradient column. Each lower band was dried on a smooth-surface agar plate, transferred to a specimen grid by the pseudoreplica method with collodion, and then shadowed with platinum-palladium alloy. The presence of a number of complete EBs was confirmed by transmission electron microscopy (Hitachi H-500) at 80 kV. After washing with 30 mM Tris-HCl the EBs were resuspended in 30 mM Tris-HCl and stored at −70°C until use.

EB counting method. A round coverslip (diameter, 8 mm) was placed on an adapter, which was made with epoxy resin in our laboratory, set in a 1.5-ml Eppendorf tube. A 250-μl aliquot of an appropriately diluted EB suspension was put on the coverslip. The centrifugation conditions, such as duration of 7 min and revolution at 3,300 × *g* (8,000 rpm), were determined in a preliminary experiment. Following centrifugation in an RS-150A swing rotor (Kubota, Tokyo, Japan), the coverslip was removed and dried. After coating with platinum-palladium alloy, the EBs in 20 to 30 randomly selected fields were counted under a Hitachi S-570 scanning electron microscope at a magnification of ×8,000. At this magnification, the ratio between one field in the screen and the whole area of the coverslip was 1:228,062.9. The actual number of EBs in the suspension was determined from the product between the average EB number per field and the dilution factor.

Immunolabeling of LPS on the surface of intact EBs and EB envelopes. Electron microscopy revealed that the EB preparations of the L_2 and TW-183 strains were mixtures of intact EBs and a few EB envelopes. Since the target antigen of the IDEIA kit is the LPS of chlamydial bodies, it was necessary to confirm the presence of LPS on the EB envelopes. Matsumoto (15) previously reported the presence of LPS on EB envelopes as well as on intact L_2 EBs prepared by a similar purification method. To confirm the presence of LPS on intact EBs and EB envelopes of the TW-183 strain prepared by the new purification method, immunogold labeling was carried out by the method of Birkelund et al. (1). Briefly, EBs mounted on a grid covered with a collodion membrane were exposed to anti-LPS monoclonal antibody (supplied by K. Hirai, Faculty of Agriculture, Gifu University, Gifu, Japan) and were then exposed to protein A-gold (diameter, 5 nm; Amersham Co.) solution. After several washings, the specimen was examined with an Hitachi H-500 transmission electron microscope at 80 kV.

Evaluation of the sensitivities of the IDEIA kit and DNA

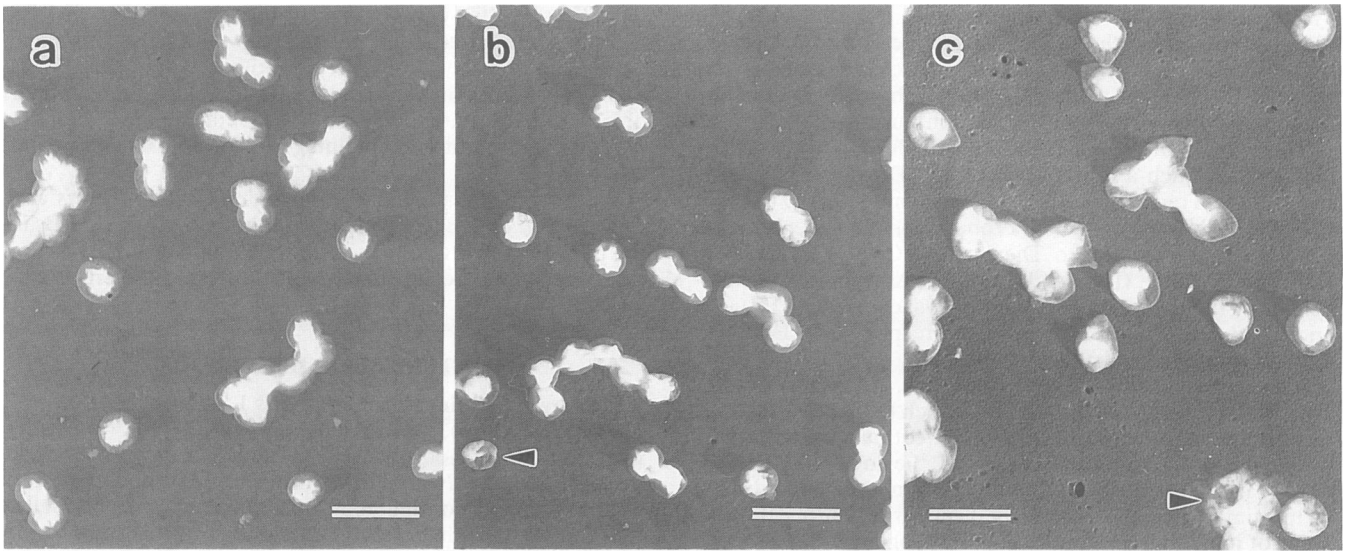

FIG. 1. Shadowed EBs of different strains in purified preparations. (a) *C. psittaci* Cal 10. (b) *C. trachomatis* L_2. (c) *C. pneumoniae* TW-183. No envelopes are seen in the Cal 10 EB fraction, but a few envelopes (arrowheads) are encountered in the L_2 and TW-183 EB preparations. Note the pear-shaped morphology of TW-183 EBs. Bars, 1 μm.

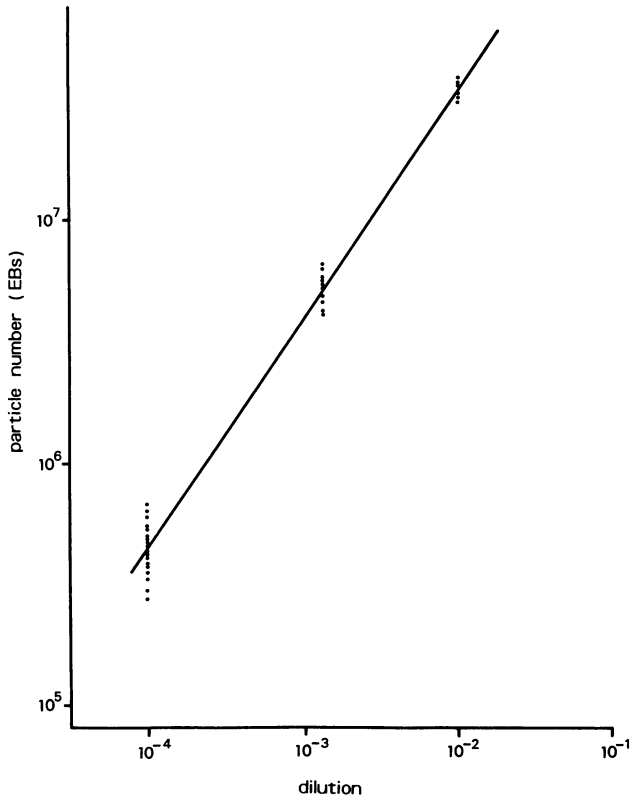

FIG. 2. Number of EBs in suspensions prepared by 10-fold dilution.

probe. In the IDEIA kit system, chlamydial LPS, which is solubilized in the transport medium contained in the kit, is captured with anti-LPS monoclonal antibody fixed in the wells of a microtiter plate and is then reacted with an enzyme-labeled, second affinity antibody. The LPS was finally assayed by the uniquely enhanced colorimetric reaction (23).

A series of EB suspensions was prepared by 10-fold serial dilution, and 0.1 ml of each suspension was added to 0.9 ml of the transport medium to prepare a second 10-fold dilution series. An aliquot of 100 μl of each dilution was used for the measurements.

The following method was used to measure the DNA probe (22, 30). A specific region of the chromosomal DNA of *C. trachomatis* was used as the labeled probe to capture the rRNA of *C. trachomatis* as a hybrid in the liquid phase. The amount of probe involved in the hybridization was detected by the chemiluminescence of the probe. Both test kits have already proved the specificities of their primary probes to chlamydiae by showing negligible interactions with other bacterial genera (13, 22). The DNA probe kit was recently modified to simplify its procedures. Therefore, we reexamined the sensitivity of the DNA probe kit.

The dilution was determined on the basis of the ratio of complete EBs and EB envelopes in the same L_2 and TW-183 EB preparations with those used in the IDEIA test kit because of the presence of EB envelopes in these EB suspensions.

All steps in the assay were performed in accordance with the instructions provided in the manuals accompanying the respective kits.

RESULTS

Purity of the EB fractions. Electron microscopy demonstrated that the Cal 10 EB suspension contained no impurities derived from the host cells or RBs and consisted of almost 100% of intact EBs (Fig. 1a). In contrast, the L_2 and TW-183 EB suspensions prepared by the newly established purification method contained both intact EBs and a few EB envelopes, which might have formed during the purification procedure (Fig. 1b and c, arrowheads). No debris derived from the host cells was encountered. On micrographs taken randomly, the ratios of intact EBs in individual samples were determined to be 96.3 and 97% in the L_2 and TW-183 suspensions, respectively.

Counting of EBs. When the EB numbers in the serially diluted suspensions were plotted on a graph, a linear relationship between the dilution and EB number per field was obtained (Fig. 2). This result suggests strongly that the method was appropriate for counting EBs in the purified preparations.

Immunolabeling of LPS on EB envelopes. By the immunogold labeling method, Matsumoto (15) reported the presence of LPS on the surface of L_2 and Cal 10 EB envelopes contained in EB fractions prepared by similar purification procedures, including DNase and RNase treatments. Matsumoto (15) also confirmed that EBs treated with the enzymes retained their morphologies and reactivities when monoclonal antibody directed to LPS was present on their surfaces. When the identical labeling method was applied to

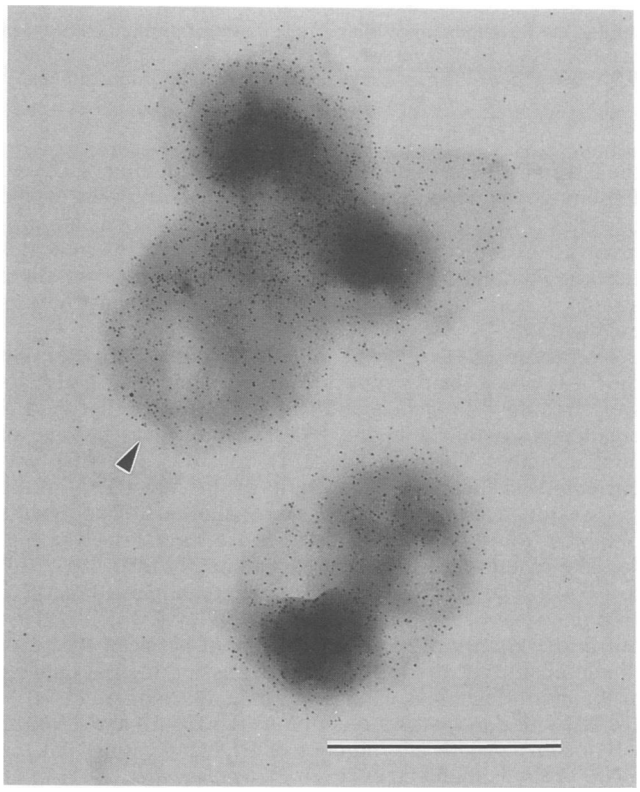

FIG. 3. Immunolabeling on the surface of intact EBs and EB envelopes of TW-183 strains. The samples were exposed to monoclonal antibody directed to chlamydial LPS and were then labeled with protein A-gold particles. Intact EBs and EB envelopes (arrowhead) are heavily labeled with the gold particles. Bar, 0.5 μm.

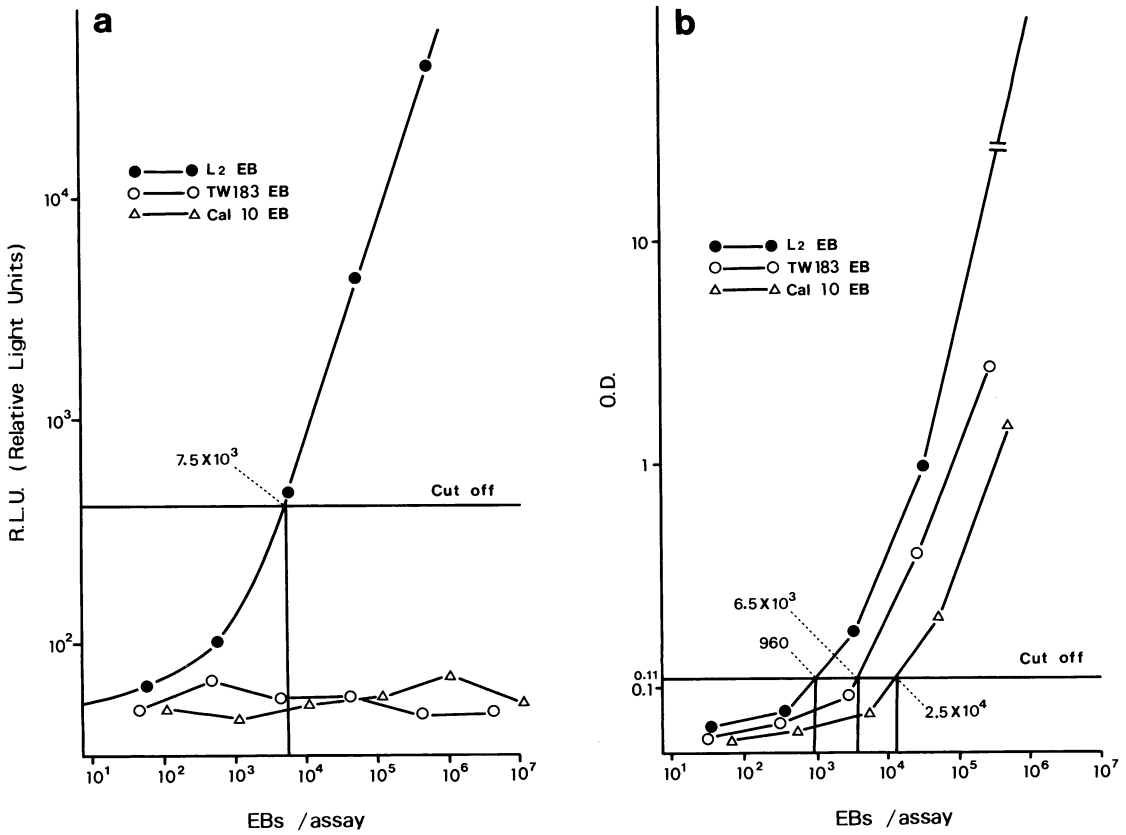

FIG. 4. Relationship of the particle count of purified EBs to reactivity with the DNA probe kit (a) and the IDEIA kit (b). (a) The L_2 EB number at the cutoff level was 7.5×10^3 per assay. (b) The number of EBs of L_2, TW-183, and Cal 10 strains at cutoff levels were 9.6×10^2, 6.5×10^3, 2.5×10^4 per assay, respectively. O.D., optical density.

the EB suspension of the TW-183 strain, both intact EBs and EB envelopes were heavily labeled with the protein A-gold particles (Fig. 3), suggesting that the EB envelopes are also involved in the reaction in the IDEIA test. This indicates that the elimination of EB envelopes from any of the three strains is not required when the IDEIA kit's sensitivity is evaluated.

Evaluation of DNA probe and IDEIA kit sensitivities. On the basis of the results reported by Matsumoto (15), all EBs were treated with DNase and RNase, to ensure that the EB envelopes contained in the EB fraction would be free of nucleic acids. The number of L_2 and TW-183 EBs was corrected on the basis of the ratio of the intact EBs in the suspension according to the serial dilutions. The results obtained from the DNA probe tests are summarized in Fig. 4a. The reaction intensity increased linearly from 1.0×10^4 to 1.0×10^6 per assay. However, the intensity at 10^2 and 10^3 EBs per assay was lower than the cutoff level which was set out in the kit. By proportional allotment between the reaction intensities at 10^3 and 10^4 EBs per assay, the EB number at the cutoff level was calculated to be approximately 7.5×10^3 EBs. In contrast, no reaction to the Cal 10 and TW-183 EBs occurred with any number of EBs, indicating that the DNA probe is highly specific to *C. trachomatis*.

Figure 4b shows the reaction intensity of the IDEIA kit to the EBs of all strains tested. However, the reaction intensity showed a decreasing order, as follows: L_2 > TW-183 > Cal 10. By a calculation performed in a manner identical to that applied to the DNA probe test, the EB numbers at the cutoff level were determined to be 9.6×10^2, 6.5×10^3, and 2.5×10^4 EBs per assay for strains L_2, TW-183, and Cal 10, respectively.

DISCUSSION

In a previous study of the IDEIA kit's sensitivity, Matsumoto (15) reported that the number of *C. trachomatis* L_2 and *C. psittaci* Cal 10 EBs at the cutoff level were 9.0×10^2 and 2×10^4 EBs per assay, respectively, and that the numbers of intracellular inclusions at 19 and 28 h postinoculation were 3.5 and 2.8 inclusions per assay, respectively, for strain L_2 and 11.5 and 7 inclusions per assay, respectively, for strain Cal 10. The DNA probe test kit was recently simplified, and the modified kit was used in the present study. Using the kit before the modification, Matsumoto and Bessho (16) examined the sensitivity of the DNA probe kit against purified L_2 EBs and intracellular inclusions at 19, 40, and 50 h postinoculation and determined 2.1×10^4 EBs per assay and approximately 10 inclusions per assay at 19 h and 5 inclusions per assay at 40 and 50 h postinoculation. The results indicated that the LPS contained in both chlamydial bodies, EBs and RBs, is reactive in the kits tested, although the numbers were different between chlamydial species and chlamydial bodies (EBs and RBs), and that the DNA probe is sensitive not only to the EBs but also to the RBs of *C. trachomatis*. The EB numbers determined in the previous studies are roughly consistent with those obtained in the present study, although the EB numbers in the previous

studies tended to be fewer than those in the present study. In the previous studies, the EB number was counted by the pseudoreplica method (17) in which the EBs that were put on a smooth agar surface were transferred onto a collodion membrane, stained by the direct immunofluorescent-antibody method, and then counted under a fluorescence microscope. Repeated experiments showed that constant reproducibility in the recovery of EBs from the agar surface was not provided, suggesting the reason why the previous EB numbers were fewer than those in the present study of the IDEIA test kit. We have established a new EB counting method, by which the linear relationship between the dilution and EB number is regularly obtained. Therefore, the EB numbers determined by the method established in the present study seemed to be reliable. The EB number (7.5×10^3 EBs per assay) determined in the DNA probe test was about one-third of that in the previous study (2.1×10^4 EBs per assay). Such an improvement in the sensitivity of the DNA probe kit might be due to the modification in the experimental procedures of the DNA probe kit.

As reported previously, *C. trachomatis* RBs were also detectable by the test kits (15, 16). However, RBs are varied in size, noninfectious (14), and fragile to the mechanical shock (29). These facts, together with the ready inactivation of EBs under the laboratory conditions (24), suggest again the appropriateness of sensitivity evaluation with the EB number used as the test parameter.

Although the DNA probe was species specific, it showed only about one-eighth the sensitivity of that of the IDEIA kit against *C. trachomatis* L_2. The higher sensitivity of the IDEIA test kit might be due to the amplification of the colorimetric reactions in the kit (23). Furthermore, this may be a main reason why the IDEIA test kit is applicable on urine samples (18, 21). However, the results obtained with the IDEIA test kit confirmed the fact that chlamydial species in positive specimens cannot be identified.

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